Metabolic Profiling of Potato Cultivars Varying in Horizontal Resistance to Late Blight, *Phytophthora infestans*.

By

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Your file Votre référence
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Our file Notre référence
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To my wife, children, supervisor, and friends

ABSTRACT

Potato is one of the most important crops grown in Canada and all over the world. Late blight caused by *P. infestans* is one of the major diseases of potato and is mainly managed by fungicides application. The extensive use of fungicides not only causes adverse effects on the environment but also accelerates the development of resistance in this pathogen. Horizontal resistance is considered as the best choice to control *P. infestans* as it is durable over years. Breeding for durable resistance requires evaluation of hundreds of breeding lines in greenhouses and in the field. This is usually done by testing several epidemiological parameters such as infection efficiency, lesion size, latent period, and area under disease progress curve (AUDPC). These methods are time-consuming and expensive. The present study reports standardization of metabolic profiling protocols and exploration of metabolic profiling based on GC/MS as an additional tool to discriminate resistance in potato against late blight. Potato cultivars varying in horizontal resistance against late blight have been inoculated with water or the pathogen and more than 100 metabolites have been tentatively identified by GC/MS. Univariate analysis has been used to identify several pathogenesis related (PR) and defense related (DR) metabolites that have potential for application as resistance biomarker metabolites. Multivariate analysis of the abundances of metabolites (the mass spectral (MS) ion trap detector outputs were obtained using Saturn Lab Software Version 5.52 and these abundances are positively proportional to the concentration of mass ions of metabolites) in cultivars were mainly used to identify pathogenesis and resistance functions. Following pathogen inoculation, several metabolites such as amino acids, organic acids, fatty acids and sugars, were significantly increased in abundances, especially in the resistant cultivar. Other metabolites such as phenylalanine, tyrosine,

shikimic acid and malonic acid detected here are well known for their direct participation in the shikimic acid, the phenylpropanoid, and the malonic acid metabolic pathways. These pathways lead to the production of several defense metabolites including antimicrobial compounds including phenolics, flavonoids and phytoalexins. The metabolic profiling technology developed here has the potential application for screening of potato breeding lines for horizontal resistance against late blight.

RÉSUMÉ

La culture de la pomme de terre est l'une des plus importantes au monde ainsi qu'au Canada. Le mildiou est l'une des principales maladies affectant la pomme de terre. Cette maladie, causée par *P. infestans*, est principalement contrôlée par l'application de fongicides. L'utilisation extensive des fongicides est néfaste pour l'environnement et contribue au développement de souches résistantes. La résistance horizontale est considérée comme le meilleur choix pour contrôler *P. infestans*. La sélection pour la résistance durable requière l'évaluation de centaines de lignées et elle s'effectue généralement en testant plusieurs paramètres épidémiologiques tels l'efficacité de l'infection, la grandeur des lésions, la période de latence ainsi que la mesure de la surface sous la courbe de progression de la maladie (AUDPC). Ces méthodes exigent beaucoup de temps et sont très dispendieuses. Nous avons exploré le profilage métabolique basé sur le GC/MS en tant qu'outil pour différencier les degrés de résistance de la patate au mildiou. Des protocoles ont été standardisés à cet effet. Des cultivars de pomme de terre ayant des degrés de résistance horizontale différents au mildiou ont été inoculés avec de l'eau ou avec le pathogène et plus de 100 métabolites ont été identifiés par GC/MS. L'analyse univariée a été utilisée afin d'identifier plusieurs métabolites biomarqueurs associés au pathogène (PR) et à la résistance (RR). L'analyse des mesures conjointes de l'abondance des métabolites dans les cultivars a été principalement utilisée pour identifier des fonctions de pathogenèse et de résistance. Suite à l'inoculation avec le pathogène, plusieurs métabolites étaient présents en plus grande quantité tels des acides gras, aminés organiques et des sucres et ce, spécialement dans les cultivars résistants. Nous avons aussi détecté des métabolites reconnus pour leur participation dans les voies métaboliques de phenylpropanoide, de l'acide shikimique et malonique tels que la phenylalaline, la tyrosine, l'acide shikimique et malonique. Ces voies mènent à la production de plusieurs métabolites de défense incluant les composés antimicrobiens du genre phénoliques, flavonoïdes et plusieurs phytoalexines. La technologie du profilage métabolique que nous avons développée pourrait être appliquée à grande échelle pour la sélection de lignées de patates ayant une résistance horizontale au mildiou.

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CONTRIBUTIONS OF AUTHORS

In this thesis, there are three studies and manuscripts (chapters: 3, 4, and 5). A manuscript of chapter 3 was co-authored by Dr. A. C. Kushalappa, Dr. W. D. Marshall, Dr. S. O. Prasher and Dr. K. Al-Mughrabi. I planned the work, performed the experiments, analyzed the data and wrote the manuscript. Dr. Kushalappa supervised the work providing valuable suggestions at different stages of this study and preparation of the manuscript. Dr. Prasher and Dr. Marshall provided the GC/MS expertise and Dr. Al-Mughrabi helped with cultivar selection and provided the potato tubers for this study.

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CHAPTER 1

INTRODUCTION

Potato is the fourth largest crop produced in the world after maize, wheat and rice. The total world production of potatoes in 2005 was 323 million metric tonnes and the total planted areas with this crop was around 18.6 million ha (FAOSTAT, 2006). In Canada, 161.5 thousand ha of potato were planted in 2005 with a total production of around 4.29 million tonnes. Prince Edward Island is the leading province with a total production of 1.10 million tonnes and Quebec came in fifth place with a total production of 464.5 thousand tonnes (Statistics Canada, 2006).

There are more than 150 registered potato cultivars in Canada. The most popular varieties planted for frying purposes are Russet Burbank and Shepody while Superior and Atlantic, are the main chipping verities. The most popular table cultivars include: Russet types, Superior, Kennebec, Norland and Yukon Gold. Around 55% of the potato grown in Canada is processed. The total consumption of potato per capita in Canada was 72.7 Kg in 2003 (Agriculture and Agri–Food Canada, 2005).

The potato plant is vulnerable to many insects and pathogens. *Phytophthora infestans* (Mont.) de Bary, the causal agent of late blight disease in potatoes, is one of the most destructive pathogens of potato, especially after the emerging of the A2 mating types. The extensive use of fungicides, especially systemic fungicides, not only increased the cost of the crop production and harmful effects on the environment but also enhanced fungicide resistance.

Breeding for vertical resistance that is controlled by major R-genes has been the most common procedure for many years to manage late blight. At least eleven R-genes have been identified against late blight and used in potato breeding programs (Wastie 1991). Unfortunately, this type of resistance is not durable and has been broken down by the appearance of new races of the pathogen. Horizontal resistance is more durable (Peters et al., 1999) and several Quantitative trait loci (QTLs) have been mapped on the 12 chromosomes of potato. The strongest QTLs associated with the horizontal resistance against *P. infestans* were found on chromosomes XII, V, III, VIII, X, IV, and II, respectively (Sliwka, 2004). Though quite high levels of horizontal resistance have been detected, the progress made in transferring horizontal resistance to cultivated potatoes has been very limited because of the difficulty of breeding for polygenic traits (Evers et al., 2003) and lack of tools to evaluate quantitative resistance phenotypes. In segregating populations though the very resistant and very susceptible disease plant-pathogen interactions are consistent among trials over years, the intermediate reactions are quite inconsistent (Haynes et al. 2002). Quantitative resistance in potato against late blight has been measured based on multiple epidemiological disease parameters such as infection efficiency, latent period, lesion sizes, amount of sporulation and area under the disease progress curve (Carlisle et al., 2002). However, these measurements are time-consuming and expensive for use in breeding programs. Plant breeders are, however, not ready to quantify all these parameters and are looking for cost-effective tools that also would reveal the mechanism of resistance to identify phenotypes varying in quantitative resistance.

Metabolomics is an evolving field of systems biology (Fiehn, 2002; Hall et al., 2002; Sumner 2003; Bino et al., 2004). Metabolic profiling has been used to study genetically modified traits (Fiehn 2000b; Roessner et al., 2001a,b; Mungur et al., 2005), to discriminate metabolites produced by leaves, stems and roots of *Medicago truncatula* (Duran et al., 2003), biotic and abiotic stressed *Medicago truncatula* (Broeckling et al, 2005), and to discriminate wheat cultivars varying in the horizontal resistance to fusarium head blight (Hamzehzarghani et al., 2005). Following pathogens attack, plants produce several pathogenesis/defense related compounds including pathogenesis related (PR-) proteins, signal molecules, phytoalexins, to deter these pathogens (Osbourn 1996a; Kombrink and Schmelzer, 2001; Taiz and Zeiger, 2002). Metabolites are the end products of genes and several of them are produced in plants in response to pathogens attack. These metabolites can be identified and used as resistance biomarkers for screening cultivars/lines on large scales in potato breeding programs.

In potato breeding, the breeders would like to know the functions of genes to pyramid suitable genes. Metabolic profiling can be used not only to screen breeding lines but also would assist in better understanding of gene functions as they are the end points of metabolism. Accordingly, our general objective was to develop a gas chromatography and mass spectrometry (GC/MS)-based technology for metabolic profiling of potato cultivars varying in resistance against *P. infestans* and to use the metabolic phenotyping as a tool for high throughput screening of potato breeding lines/cultivars for resistance to late blight.

1.1. Research Hypothesis:

Phytophthora infestans is a hemibiotroph (starts infection as a biotroph then becomes a necrotroph) where it secretes little enzymes at the early stages of infection to minimize disturbance of defense responses of plant. Biotrophic pathogens are characterized by producing little lytic enzymes and are able to suppress the defense responses of the host for long periods (Mendgen and Hahn, 2002). As the pathogen turns to the necrotroph phase, it starts to secrete several digestive enzymes and toxins that result in the death of several cells leading to the appearance of necrotic lesions in the diseased plants. In response to the pathogen attack, resistant cultivars produce several antimicrobial and defense related metabolites by activating several metabolic pathways. Accordingly, it is hypothesized that potato cultivars with different levels of horizontal resistance would vary in their metabolic profiles following the inoculation by *P. infestans*. Using GC/MS, several metabolites can be analyzed and used to discriminate potato cultivars with different levels of horizontal resistance to *P. infestans*. Extraction of both polar and non-polar metabolites separately would increase the efficiency of compound separation by GC/MS, identification, and eventually the total number of detected metabolites.

1.2. Research Objectives

The general objectives of this research were to develop metabolic profiles of some potato cultivars varying in horizontal resistance to late blight, *P. infestans*, and to relate them to levels of resistance to identify resistance biomarker metabolites that discriminate resistance for their future exploitation in screening of breeding lines. The specific objectives are:

1. To standardize a protocol for metabolic profiling of potato leaves and to find out if potato cultivars with contrasting levels of horizontal resistance to leaf infection by *P. infestans*, US-8 genotype, vary in their metabolic profiles (Study I).
2. To study the temporal dynamics of potato metabolites and their plausible pathways of production with the progress of the pathogen and to find the most appropriate time length of incubation for the highest expression/accumulation of metabolites after the inoculation by *P. infestans* (Study II).
3. To explore if metabolic profiling can be used to phenotype potato cultivars varying in horizontal resistance against late blight, and to identify resistance biomarker metabolites for potential application in screening breeding lines for horizontal resistance against *P. infestans* (Study III).

CHAPTER 2

GENERAL LITERATURE REVIEW

2.1. Potatoes

2.1.1. Potato origin and classification

It is believed that potatoes originated, and were cultivated for more than 7,000 years near the Andes of Southern Peru and Northern Bolivia (Hawkes, 1994). Potatoes were first introduced to Europe in the 16th century and after adaptation to long day conditions, it became an important source of food (Beukema and Zaag, 1990). Potatoes were introduced to North America from Bermuda during the second half of the 17th century (Hawkes, 1994).

Potato belongs to the large and diverse genus, *Solanum*. More than 228 wild species and seven cultivated species of potato have been reported. These species have a basic number of 12 chromosomes (x=12), ranging from diploid (2n=2x=24) to hexaploid (2n=6x=72). The cultivated species include: *S. stenotomum* (2x), *S. ajanhuiri* (2x), *S. phureja* (2x), *S. chaucha* (3x), *S. juzepczukii* (3x), *Solanum tuberosum* (4x) and *S. curtilobum* (5x). In addition, the species *S. tuberosum* is subdivided to two subspecies *S. tuberosum* subsp. *andigena* and *S. tuberosum* subsp. *tuberosum* (Hawkes, 1994). Dean (1994) reported another species *S. goniocalyx* (2x) to the cultivated potatoes to increase the number to eight. According to Beukema and Zaag (1990), most of the commercial potato cultivars are "4x" and derived from *S. tuberosum* subsp. *andigena*. Potato is a good source of carbohydrates and contains significant amounts of proteins, vitamins, dietary fibers and minerals. Carbohydrates constitute about 75% of the total dry matter

of the tubers and starch is the major component and the main energy source (Burton, 1989). Proteins compose 1.6-2.1% of the total fresh weight of potato and their concentrations vary between different cultivars and even within the same cultivar and they are more abundant in the tuber cortex than the pith. Patatin is the major soluble glycoprotein that contributes around 40 % of the total soluble proteins in the potato tubers. Other important soluble proteins include: Globulin (tuberin), albumin (tuberinin) and glutelin. Potato vitamins belonging to the B and C groups are the most important in their contribution to human daily uptake requirements (Burton, 1989).

2.1.2. Potato breeding

Potato breeding is difficult and time consuming. Before a cultivar is released to the market, it undergoes extensive tests for desirable trait selections. It takes usually about 15 years to produce a successful cultivar. In potato breeding programs, high yield, good adaptation, good storability, acceptable quality and acceptable levels of disease resistance are the most important criteria to be taken into consideration (Beukema and Zaag, 1990). Cultivars that are planted for chip processing usually have round shaped tubers, with a specific gravity of 1.080 to 1.095, free of defects and have a light color upon frying. Cultivars with lower specific gravity produce lower chip yield, takes more time to be fried and absorbs more oil. On the other hand, cultivars with high specific gravity are more sensitive to bruising during handling (Rowe, 1993; Dean, 1994).

The tetraploid potato cultivars have the highest yield potential; therefore, most of the breeding programs are devoted to hybridize for the tetraploid. Unfortunately, as the number of the homologous chromosomes increase, genetic variation (Vg), and in the

presence of environmental variation (Ve) results in a high phenotypic variations (Vp) (Beukema and Zaag, 1990). In addition to the remarkable variation in the *Solanum* gene pool, a high degree of ploidy increases the genetic diversity and increases the difficulty of producing new cultivars. In conventional breeding programs, hundreds of tests and crosses are conducted to find few offspring that are better than their parents (Dean, 1994). Therefore, the study of inheritance in potatoes is difficult. In the presence of four homologous chromosomes, there are five different possibilities for the genotypes at each locus, one homozygous and 4 heterozygous i.e. AAAA, AAAa, AAaa, Aaaa, and aaaa. These are known as quadriplex, triplex, duplex, simplex and mulliplex, respectively. There are also three possible types of gametes: AA, Aa, and aa (Beukema and Zaag, 1990; Gebhardt and Valkonen, 2001).

2.2. Potato pests and diseases

2.2.1. General Pests and diseases

Many pests and pathogens attack potato during its production and storage. Colorado potato beetle (*Leptinotarsa decemlineata*), the European corn borer (*Ostrinia nubilalis*), the green peach aphids (*Myzus persicae*), the flea beetles, the two-spotted spider mites (*Tetranychus urticae*), potato psyllid are among the most important pests that attack potato. Potato Virus Y (PVY), Potato Virus A (PVA), (PVM), (PVS) and Alfalfa Mosaic Virus (AMV) are examples of the most important viruses that attack potatoes. In addition, root knot nematodes (*Meloidogyne* spp.), root cyst nematodes (*Globodera* spp.), root-lesion nematodes (*Paratylenchus* spp.) and many other nematodes can cause severe damages when they attack potatoes. Blackleg (*Erwinia*

carotovora subsp. *atroseptica*) and tuber soft rot (*Erwinia carotovora* subsp. *Carotovora* = *Pectobacterium cartovorum* subsp *cartovorum*) are the most destructive bacterial pathogens that infect potato. Moreover, potato is vulnerable to the attack of many fungi. *Sclerotinia, Rhizoctonia, Pythium*, and *Phytophthora* are very destructive pathogens and widely spread all over the world (Rowe, 1993; Dean, 1994). Late blight and Potato Virus Y (PVY) are the major problems of potato grown in temperate climates (Gebhardt and Valkonen, 2001).

2.2.2. Late blight

Potato late blight *Phytophthora infestans* is one of the most destructive pathogens that attack potato (Goodwin et al., 1994). Historically, this pathogen caused the famous famine that spread in Ireland during the 1840s (Gebhardt and Valkonen, 2001) and resulted in the death of more than one million people and the immigration of more than 1.5 million to North America (Rowe, 1993). According to Duncan (1999), worldwide losses caused by *P. infestans,* including the cost of control measures, exceed US$ 3 billion annually.

P. infestans belongs to the kingdom Chromista, Phylum *Oomycota*, Class *Oomycetes*, order *Peronosporales*, and Family *Peronosporaceae* (Birch and Whisson, 2001). Oomycetes are very close to diatoms and brown algae and are recently been classified under the new kingdom, Stramenopiles (Baldauf, 2003; Torto-Alalibo, 2005; Birch et al., 2006). *Phytophthora* and other members of the class oomycetes are different from fungi in that they contain little chitin in their cell walls (Erwin and Ribeiro, 1996). This genus contains many species that can attack a wide variety of hosts like

ornamentals, fruit and forest trees and causes serious diseases such as root and lower stem rots, trunk cankers, twig blights and fruit rots. It also can attack different vegetable including strawberries, potatoes, eggplants, tobacco, tomato and many other crops (Birch and Whisson, 2001; Agrios, 2005). Some important species of this genus include: *P. cambivora* the causal agent of the crown rot and trunk canker of cherry; *P. parasitica* that cause the trunk rot of citrus trees; *P. palmivora* that causes the the black pod disease of cacao; *P. fragaria* the causal agent of red stele root rot of strawberry. Other important spesies include: *P. cacotorum, P. cinnamoni, P. citrophthora, and P. syringae* (Agrios, 2005). *P. infestans* can infect plant leaves, stems and potato tubers in the field and during storage (Peters et al., 1999) and can complete the asexual life cycle from infection to the production of sporangia in less than 5 days (Fry and Goodwin 1997). Moreover, *P. infestans* provides an entry point for other pathogens like soft rot bacteria (Fry and Goodwin, 1997).

P. infestans is a heterothallic pathogen requiring the A1 and the A2 mating types for sexual reproduction (Daayf and Platt, 1999; Peters et al., 1999; Birch and Whisson, 2001; Stromberg et al., 2001). Before the 1980's, the A2 mating type was restricted to Mexico (Fry et al., 1993). It is hypothesized that the A2 mating type of this pathogen was first introduced to the USA in the late 1980s and to Canada in the early 1990s (Deahl et al., 1991; Fry et al., 1993; Goodwin et al., 1994). Since then, the A2 became more dominant in many parts of Canada (Goodwin et al., 1998; Peters et al., 1998; Peters and Platt, 1999, Daayf et al., 2000; Daayf and Platt, 2002). The A2 type also has the ability to sexually interbreed with the A1 mating type to produce oospores, the over wintering structures (Fry et al., 1993; Peters et al., 1998; Daayf et al., 2000). The

oospores can tolerate adverse weather conditions and can survive in the soil for months or years (Birch and Whisson, 2001) and they are an early source for infection in the field (Stromberg et al., 2001). When germinating, the oospores produce new offspring of either A1 or A2 mating types (Drenth et al., 1995). Sexual reproduction increased the diversity of *Phytophthora* and resulted in high levels of variation of this pathogen (Fry and smart, 1999; Flier et al., 2003). Examples of some of the reported A1 genotypes are: US-1, US-6, US-11 (g-11), US-12, US-16 and US-17. The A2 genotypes include: US-2, US-7, US-8, US-13, US-14, US-15, US-18, US-19, US-29, g26, g29, g30, g40, g41, and g42 and many others (Reported in Goodwin et al., 1998; Peters et al., 1999; Daayf et al., 2000; Wangsomboondee et al., 2002).

Phytophthora has one of the largest genomes of all microbes and is estimated to be around 240 Mbp (million base-pairs) (Bos et al., 2003). DNA fingerprinting with the RG57 probe, for example, was used for genotype identification (Wangsomboondee et al., 2000 cited in Wangsomboondee et al., 2002). Recently, the genetic linkage map studies of *P. infestans* using Amplified Fragment Length Polymorphism (AFLP) and Restriction Fragment Length Polymorphism (RFLP) enabled the positioning of six dominant Avr loci on the genome map. Avr4 was placed on linkage group (LG) A2-a, Avar1 on LG IV, Avr2 on LG VI, and Avar3, Avar10 and Avar11 were tightly linked and mapped on LG VIII (van der Lee et al., 2001).

P. infestans is a hemibiotroph. After the inoculation of different plants with *P. infestans* (susceptible cultivars of *Solanum tuberosum*, cultivars with different combination of r-genes, partly resistance, complete resistance *Solanum berthaultii* clone 9, and non-host plants like *Nicotiana tabacum* and *Arabidopsis thaliana*), results showed

that hypersensitive response (HR) expressed at different speeds and magnitudes and was highly associated with all interactions. The HR in non-host plants was very strong and no biotrophic phase was seen and completed in most cases within 22 hours after inoculation (HAI). In completely resistant plants i.e. *Solanum berthaultii* clone 9, the HR was slower and completed in 46 hours and no biotrophic phase was seen. In plants with high partial resistance i.e. *Solanum berthaultii* clone 11, the HR was slower than the complete resistant and 16% of the infected lesions had a very slow lesion growth rate (LGR) of 1.2 mm per day. The hyphae expanded out of the epidermal cells to the mesophyll that exhibit a HR and postponed the pathogen. The plants with lower partial resistance showed a less effective HR and higher infection efficiency (IE) of 86%, and the LGR was 2.4 mm per day. At 46 HAI, the hypha in the partially resistance cultivar, Robijn, escaped the hypersensitive lesion and induced biotrophic response in the mesophyll cells and sporangiophores were formed with fashion similar to susceptible cultivars. In susceptible plants, the major responses included a biotrophic phase and the HR was very slow. At around 16 hours, or later, after inoculation of the susceptible cultivars Bintji and Bildtstar (both susceptible), the pathogen was able to penetrate the epidermal cells of potato and to produce intracellular infection vesicles. After 22 hours, the pathogen hyphal branches were expanded in the intercellular spaces of the mesophyll. This phase represents the early biotrophic stage and 1-2 haustoria were seen inside each of the infected parenchyma cells. In later stages of invasion, hyphae hardly produced any haustoria and at around 46 h, the sporangiophores started to emerge through the plant stomatas and the infected cells started to develop necrosis. During this phase, the pathogen expansion was high and the LGR was 4.0 mm per day. In this

compatible interaction and in very rare cases only one epidermal cell showed HR response. At 72 h, the leaf necrosis was easily seen by the naked eye (Vleeshouwers et al., 2000).

2.3. Disease management

2.3.1. Chemical control

In general, Late blight is mainly managed by the use of fungicides. In Europe, an average of eight fungicide applications is used to control this pathogen. In some years, this number increases to reach 30 applications (Duncan, 1999). In addition to the high cost of fungicides, they are not safe and pollute the environment. Moreover, *P. infestans* has been able to develop resistance against many fungicides in many areas and this makes the fungicide application non profitable in many situations.

The A2 mating types were found to be more aggressive, and more resistant to fungicides, especially metalaxyl than their A1 counterparts (Fry and Smart, 1999). Medina et al., 1999 showed that the newly introduced US-8 genotype was more aggressive and caused more damage on tubers than the US-1 genotype. In the United States and Canada, isolates of the A2 mating type collected in the years 1993-1996 were found to be moderately resistant or resistant to metalaxyl treatments and the isolates belonging to the A1 mating type were found to be more diverse and to be susceptible, moderate, or resistant to this fungicide (Goodwin et al., 1998). In Canada, it has been reported that the percentage of the US-8 genotype increased from 49 to 74% in the years 1994 and 1995, respectively. In both years, most of the collected US-8 genotypes were insensitive to metalaxyl control (Peters et al., 2001). Moreover, in 1996, the A2 mating

type was found to be more dominant than A1 mating types in all Canadian provinces except British Columbia and the new A2 mating types were more resistant to metalaxyl treatments (Peters et al., 1998). In 1997, 364 isolates of *P. infestans* were collected from different Canadian provinces of which 292 isolates were found to belong to A2 mating type. Of the 364 isolates, 14, 70 and 16% were found to be susceptible, intermediate and resistant to the fungicide metalaxyl, respectively (Daayf and Platt, 1999). In 1996-1998, samples collected from different Canadian provinces were found to have the A2 mating type (Daayf et al., 2000). During 1996-1998, In *Vitro*, US-8 isolates collected from potato in the years 1995 and 1998 were found to be less sensitive to mancozeb and metalaxyl-m treatments than those collected in the years 1994, 1996 and 1997 (Daayf and Platt, 2002). In addition to its ability to infect potato, samples collected from potato or tomatoes were found to have the ability to infect both plants. Thirty-two isolates of genotypes US-8 and US-11, the main genotypes reported in Canada, were found to have the same levels of infection on potato leaves while the US-11 was found to be more aggressive on tomato leaves (Daayf and Platt, 2003).

2.3.2. Breeding for disease resistance to *Phytophthora infestans*

Breeding for disease resistance against *P. infestans* became the primary goal of many breeding programs (Swiezynski and Zimnoch-Guzowska, 2001). Vertical resistance (monogenic resistance) is a race-specific resistance, based on "gene-for-gene" interaction. It is controlled by a single major R-gene and is mainly characterized by the activation of the hypersensitive reaction (HR) that causes the death of both the plant cell and the attacked pathogen (Vleeshouwers, et al., 2000). In the past, breeding programs

have mainly considered vertical resistance because of the relative ease of transferring genes from closely related wild *Solanum* species. However, vertical resistance increases the selection pressure and the build up of new aggressive races that can infect the resistant cultivars within a few years. This can be seen in the field as "boom-and-bust cycles" (Keller et al., 2000). Lately, Isolation and cloning of some of the R-genes has been reported (Song et al., 2003; van der Vossen et al., 2003; Huang et al., 2005) and the RB gene (*Rpi-blb1*) from *Solanum bulbocastanum* is believed to have durable resistance in the field (Song et al., 2003). Recently, many new races of *P. infestans* have been found in North America. This is due to the occurrence of both A1 and A2 mating types that can reproduce sexually. The increased numbers of new races made breeders consider the horizontal resistance (polygenic resistance) in the breeding programs. This resistance is more durable than the vertical resistance due to the polygenic nature of inheritance (Simmonds and Wasie, 1987). This resistance consists of several minor genes of which none can give absolute resistance to *P. infestans*. The minor genes work together to slow the rate of colonization and reduce the rate of sporulation of the pathogen (Sliwka, 2004). However, the progress made in transferring horizontal resistance to cultivated potatoes has been very limited because of the difficulty in breeding for polygenic traits (Evers et al., 2003). Despite the high numbers of wild species of potato, only a few of them are used in the breeding programs for disease resistance because of the interference of the wild traits with the desirable traits already existing in cultivars. Several generations of backcrossing and selections are required before getting acceptable cultivars (Gebhardt and Valkonen, 2001). Recently, significant progress has been made in the area of genetics of plant disease resistance.

Gene markers have been used to map and position disease resistance genes in the potato genome. Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), Simple Sequence Repeat (SSR), and Polymerase Chain Reaction (PCR) based markers have been used to map R-genes and Quantitative Trait Loci (QTLs) on the 12 chromosomes of the potato genome (Gebhardt and Valkonen, 2001; Sliwka, 2004). For example, nineteen single dominant R-genes have been reported to be effective against viruses, nematodes, and fungi. (Reviewed by Gebhardt and Valkonen, 2001). At least 11 single R-genes for resistance to *P. infestans* have been introduced into cultivated potato from the wild potato *Solanum demissum* (Wastie, 1991). These R-genes induce hypersensitive reactions (HR) upon infection by specific races of *P. infestans*. For example, the R1 locus is mapped onto chromosome number V, the R2 on chromosome IV, and R3, R6, and R7 on chromosome XI. In addition, the R_{ber} and R_{blc} resistance genes against *P. infestans* originated from *S. berthaultii* and *S. bulbocastanum* have been mapped onto chromosomes X and VIII, respectively (Reviewed by Sliwka, 2004). In the potato DNA marker maps, it was possible to identify several loci having cluster of R-genes that confer resistance to different races of *P. infestans*. For example, the three major R-genes: *R3a, R3b, R6,* and *R7* are clustered on the distal segment of chromosome XI by the marker locus *TG105(a)*. Another cluster was located on chromosome V (Gebhardt and Valkonen, 2001, Sliwka, 2004). Different *Pi* (*P. infestans*) QTLs for foliage and tuber resistance were mapped on chromosomes I-XII (Reviewed by Gebhardt and Valkonen, 2001; Sliwka, 2004). The strongest QTLs associated with resistance against late blight were found in chromosomes XII, V, III, VIII, X, IV, and II, respectively (Sliwka, 2004). Though QTL with disease resistance has

been mapped, the interactions of such traits with other undesirable traits make transfer of such traits difficult. For example, the QTLs for late maturity and foliage resistance to *P. infestans* are mapped in the same position on chromosome V and found to be highly associated (Collins et al., 1999, Ghislain, 2001). Visker et al., (2003) speculated that these two QTLs might be the same gene with pleiotropic effect on both traits. Though such technologies appear promising, cloned cultivars with durable, QTLs resistance to late blight are yet to be developed. In general, cloning or introducing one gene does not always mean that the plant will become resistant. Other factors, like the environment, are important in the expression of these genes. In several crops, cloning of a single gene or QTLs might have an antagonistic effect on the expression of other genes. For example, a mutant barley cultivar with an *mlo* gene gives durable resistance to powdery mildew but this gene causes a small decrease in the yield and makes barley more susceptible to the fungus *Magnaporthe grisea* (Stuiver and Custers, 2001). During the crossing of durum wheat and *Triticium tauschii* to produce a synthetic wheat cultivar, the leaf rust resistant gene *Lr23* introduced from durum wheat was suppressed by another gene located in the genome of *T. tauschii*. The plant was found to be susceptible to the leaf rust fungus. In contrast to suppressor genes, complementary genes might be needed to activate the plant defense response. For example, the presence of both *Lr27* and *Lr31* genes in wheat is essential to provide resistance to leaf rust. In wheat, the stem rust genes *Sr9b, Sr10* and *Sr11* are not effective against the stem rust strain 15B-1 but they modify the plant *Sr7a* gene and make wheat resistant to this strain (Keller et al., 2000).

A new trend in studying disease resistance is cloning of genes that are responsible for the production of plant signal compounds such as salicylic acid, jasmonic acid, and ethylene. Treatment of the plant with one or more of these signaling compounds causes the production of antifungal proteins, phytoalexins, and many protective enzymes. These signal-transduction compounds are known as "master switchers." While attempting to generate a broad-spectrum disease resistance, group teams at Syngenta-MOGEN with the collaboration with other groups were successfully able to create transgenic tomato and tobacco plants. Their work was focused on using the hypersensitive response to create a broad-spectrum disease resistance in plants. This was done by transferring a pathogen-derived elicitor gene to a plant and putting that gene under the control of a tightly regulated pathogen-inducible plant promoter. This gene is only activated upon the infection by the pathogen (cited in Stuiver and Custers, 2001).

2.4. Functional genomics

Genomics is the study of the structure, function and evolution of the genome of an organism. The structural genomics is concerned with studying the structure of the molecules of an organism, its genes, transcripts and proteins (Kamoun and Smart, 2005). Gene expression involves transcription and translation. In transcription, the genetic information is copied from the DNA, which is used as a template, to produce mRNA. In translation, the genetic information from the mRNA is used to produce a specific polypeptide or a protein. In functional genomics the main goal is to study, analyze, and understand the correlation between the genes and the functional phenotypes of an

organism (Bino et al., 2004). In addition to transcriptomics, proteomics, metabolomics is recommended be included as an integral part of the functional genomics studies for better understanding the functions of genes (Sumner et al., 2003).

2.4.1. Transcriptomics and Proteomics

Gene transcription results in the production of mRNAs that lead to the production of transport, storage, hormonal, defense, structural proteins or enzymes. However, the presence of mRNA does not give an indication of the type of protein that is going to be produced and weather it is going to be active or not (Campbell et al., 1996). Moreover, gene expression, as a result of post transcriptional modifications, sometimes produces more than one enzyme (isozymes that carry different enzymatic reactions under different conditions i.e. pH and temperature) (Trail and Koller, 1993). In humans, the estimated number of genes were estimated 140,000 and were estimated to produce more than 1 million proteins as a result of the post transcriptional factors i.e. one gene encodes for 7 proteins in average (Jacobs, et al., 2000). Recently, this number has been estimated to be between 20,000 and 25,000 (Stein, 2004). The transcriptome approach of using ESTs (Expressed Sequence Tags) has been used to study potato plants infected with *P. infestans* (Ronning et al., 2003) and to study the genome of *P. infestans* (Gajendran et al., 2006). It has been used to study the abiotic stress of potato plants (Rensink, 2005). Proteomics is a large-scale study of the function of all expressed proteins or a systemic analysis of expressed proteins (Jacobs et al., 2000; Pandey and Mann, 2000; Tyers and Mann, 2003; Newton et al., 2004). Proteomics gives information about the gene products regarding when and how much protein is going to be expressed.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and mass spectrometry (MS) are powerful techniques that have been used in the separation and identification of proteins (Jacobs et al., 2000). Although transcriptomics and proteomics are very useful in plants, they do not give sufficient information on the produced metabolites (Roessner et al., 2000; Fiehn 2001).

2.4.2. Metabolomics

Metabolomics was defined as a 'non-biased identification and quantification of all metabolites in a biological system' (Dunn et al., 2005). Plant metabolites are the products and the ultimate final changes in any biological system in response to genetic material and the interaction between genes and the environment (Fiehn, 2002). Primary metabolites are essential for the growth and development of the plant, while the secondary metabolites are usually associated with the defense responses in the plant against stresses and also the production of floral scent and coloration to attract pollinators (Kliebenstein, 2004). In the plant kingdom, plant metabolites are numerous and estimated to be around 200,000 (Fiehn, 2002). However, in a specific plant and at a specific time, this number is less and proportional to the size of the individual genome. For example, it is estimated the total number of genes in *Arabidopsis thaliana* is around 28,000 (Schoof et al., 2004) the total number of the primary and the secondary metabolites is about 5,000 (Bino et al., 2004). In addition to this huge number of metabolites, great diversity in the molecular weights, chemical properties and the wide range of concentrations (arranged between pM- mM) makes the study of the plant metabolome more challenging (Dunn et al., 2005).

Metabolites can be grouped as volatiles and non-volatiles. Plant metabolic volatiles have been used widely for metabolic profiling and discrimination of plant diseases. Pasanen et al. (1996) studied microbial volatile organic compounds by using thermal desorption-gas chromatography. Twelve microbial volatile organic compounds (MVOC) were identified in this study and a relation between the synthesis of mycotoxins and the relative proportions of different MVOC were detected. The toxigenic strains were found to produce more volatile ketones than the non-toxigenic strains. On the other hand, de Lacy Costello et al. (1999) used GC/MS to study the interaction between different species of bacteria and potato tubers. They were able to detect 22 unique volatiles following infection of the tubers by *Erwinia carotovora*; three volatiles associated with *Bacillus polymyxa* and one unique with *Arthrobacter*. In addition, some important volatile metabolites produced after the infection by *P. infestans* have been reported (de lacy Costello et al., 2001). Volatile metabolites analysis using GC has been used to discriminate five diseases of potato tubers (Kushalappa, et al. 2002). Huang et al. (2003) studied interactions between tobacco and the bacterium *Pseudomonas syringae* pv. *tabaci* and the total emission of some volatiles like methyl salicylate by using gas chromatography was reported. Recently, volatile metabolites have been used to discriminate several diseases of McIntosh apple (Vikram et al., 2004a,b), onion (Vikram et al., 2005) and potato (Lui, et al., 2005).

Many techniques and analytical equipments have been used for the separation and for the identification of non-volatile metabolites. No single technique can be used or recommended to study the entire plant metabolome. Thin layer chromatography (TLC), high performance liquid chromatography (HPLC), HPLC coupled with both mass

spectrometry, and nuclear magnetic resonance (LC/NMR/MS), capillary electrophoresis coupled with ultraviolet absorbance detector (CE/UV), capillary electrophoresis with mass spectrometry (CE/MS), gas chromatography-mass spectrometry (GC/MS), nuclear magnetic resonance (NMR), and many other techniques have been used in metabolic profiling and fingerprint studies were reviewed by (Fernie, 2003; Sumner et al., 2003; Dunn et al., 2005). When selecting an instrument, speed, selectivity, sensitivity and dynamic range are given as the top priorities (Fiehn, 2002; Sumner et al., 2003).

Metabolic fingerprinting and metabolic profiling are well-established techniques used to study non-volatile plant metabolites. Metabolic fingerprinting is a rapid, global screening method for analyzing crude or plant extracts samples. In this method, identification or quantification of the plant metabolites is not required (Kopka et al., 2004; Dunn et al., 2005; Halket et al., 2005). The most important devices that have been used in metabolic fingerprinting include: Quadrupole-time of flight (Q-TOF), Fourier transform-ion cyclotron resonance (FT-ICR), and Fourier transform infra-red (FT-IR) (Johnson et al., 2003; Dunn et al., 2005; Halket et al., 2005). On the other hand, metabolic profiling involves identifying and quantifying a select numbers of pre-defined metabolites in a biological system that result from a genetic alteration (Broeckling et al., 2005; Dunn et al., 2005). Metabolic profiling of plant tissues in a large scale is a recent field of study that emerged in the late 1990's (Adams et al., 1999; Katona et al., 1999) and potatoes was among the first plants to be metabolically profiled (Roessner et al., 2000 and 2001a,b). GC/MS is being used extensively in many labs because it is highly efficient, sensitive, highly reproducible, and the cost of a sample analysis is relatively cheap (Fiehn (2000a); Glassbrook, 2000; Hall et al., 2002; Sumner et al., 2003).

Different types of analyzers have been also used with GC/MS: time of flight (TOF) mass spectrometry (Fiehn et al., 2000a), ion trap (IT) mass spectrometry (Bianco et al., 2002; Tolstikov and Fiehn, 2002) and quadrupole mass spectrometry (Roessner et al., 2000 and Fiehn et al., 2000b). While studying the plant metabolites, the experimental conditions must be specified as much as possible and the metabolic profiling techniques must be fast, consistent, sensitive, and comprehensive to cover a large number of metabolites (Fiehn, 2002 and Bino, 2004). In addition, unbiased methods for simultaneous identifications and quantifications of metabolites are needed (Roessner, 2000; Fiehn, 2002). Different techniques and methods have been used for plant samples collection, handling, freezing in liquid nitrogen, storage in deep freezers at –80 °C, lypholization, extraction, chemical derivatization and ionization in GC/MS (reviewed by Sumner et al., 2003; Kopka et al., 2004; Dunn et al., 2005; Halkket et al., 2005).

Metabolic profiling has been used to identify genetically modified traits and to evaluate these traits after growing under different environmental conditions (Roessner et al. 2001b). Roessner et al. (2000) were able to identify more than 150 metabolites while studying potato tubers using GC/MS technique. Major differences were found in the concentration of the amino acids such as glutamine, proline, and arginine. In vitro microtubers were found to have higher concentrations of the amino acids compared with the soil-grown tubers. Fiehn et al. (2000b) used metabolic fingerprinting for the comparison of two homozygous ecotypes and two single gene mutants of *Arabidopsis thaliana*. Distinct metabolic phenotypes were reported for each genotype. In total, 326 distinct metabolites were identified and metabolic phenotypes were found to be more diverse within the parent's ecotypes when compared with the metabolic phenotypes of

each mutant and his parental ecotype. Duran et al., (2003) were able to discriminate between the metabolites extracted from leaves, stems and roots of *Medicago truncatula* by using metabolomics spectral formatting, alignment and conversion tools program (MSFACTs). The first two principle components (PCs) were able to explain 92% of the total variance and more similarities of metabolites detected within leaves and stems were found compared to metabolites from roots.

The metabolomics approach is potential for studying plant-pathogen interactions. Certain defense pathways and many metabolites in the host plant are expected to be activated in response to pathogen attack. Therefore, those metabolites could be used to evaluate the horizontal resistance of plants.

Although plant metabolomics has been the focus of many recent studies, this approach is still evolving (Hall et al., 2002). Many labs are using different instruments and techniques for studying the plant metabolome and this obstruct the data sharing and exchanging between labs. Metabolic profiling of a plant is highly dynamic and is found to be varied between genetically modified plans grown in different environmental conditions (Roessner et al., 2001) and even between different tissues of the same plant grown under similar conditions (Duran et al., 2003). Therefore, Minimum Information About METabolomics (MIAMET) has been proposed by many metabolome leading labs to standardize methods for experimental design, plant samples collecting, handling, storage and preparation the samples for testing. In addition, MIAMET includes recommendations for using standard protocols for the metabolites extractions (i.e. polar and non-polar metabolites) and certain protocols for metabolites derivatization before analysis. Also, when using the same instrument for sample analysis, the running

conditions of the instrument must be standardized. For example, while using GC/MS, the type of column, the injection, interface, the ion source, and the oven ramping temperatures are to be standardized. Moreover, MIAMET suggested standardization of methods for chemical identification including the usage of mass spectral libraries provided by NIST (National Institute of Standard and Technology; http://www.nist.gov/), Wiley (http://www.wileyregistry.com/) and Sigma-Aldrich (http://www.sigmaaldrich.com) for metabolites identification. Adapting MIAMET recommendations will organize and facilitate data sharing between different labs (Bino et al., 2004).

2.5. Plant defense against pathogen attack

Plants defend themselves against pathogens by the activation of different defense responses including the reinforcement of the cell wall, production of reactive oxygen species (ROS), hypersensitive reaction (HR), enzymes, PR-proteins, and the production of antimicrobial compounds such as phytoalexins (McDowell and Woffenden, 2003). Plant defense responses can be broadly classified into pre-formed/constitutive and induced, and within each into structural and biochemical (Agrios, 2005). In the following sections, some important metabolites produced by the plants will be discussed. More attention will be given to the small molecular weight metabolites that can be detected by GC/MS, and are related to plant defense.

2.5.1. Pre-formed Structural Defenses

2.5.1.1. Plant cuticle

Plant cuticle is the first barrier that is effective in many situations to defer the pathogen attack. The major components of the plant cuticle are: wax, cutin cellulose and little pectins.

2.5.1.1.1. Wax layer

The epicuticular wax is composed of relatively hydrophobic hydrocarbons. It is composed mainly of long chain alcohols, straight-chain alkanes, long chain fatty acids and fatty acid esters (Taiz and Zeiger, 2002). Many plant pathogens cannot degrade waxes, however, some fungi such as *Puccinia hordei* can (Agrios, 2005).

2.5.1.1.2. Cutin

Cutin is a polymer of long chain fatty acids that are united together by ester linkages (Taiz and Zeiger, 2002). In the plant plastids acetyl-CoA is used for the production of Lauric acid (12:0), Palmitic acid (16:0), Stearic acid (18:0) and Oleic acid (18:1). These fatty acids are then transferred to the endoplasmic reticulum (ER) for modification. Different enzymes like peroxygenase, epoxide hydrolase and cytochrome P-450 are used to convert palmitic acid and oleic acid to 10,16-Dihydroxypalmitic acid and the 9,10,18-Trihydroxy stearic acids, respectively. These modified fatty acids are then used as precursors for the cutin biosynthesis. Cutinases are extracellular enzymes that can breakdown cutin. They are glycoproteins containing 3-16% carbohydrates. They have been detected, isolated and purified from many plant pathogens (Huang, 2001). In addition to their major role in cuticle penetration, cutinases are believed to facilitate the adhesion of the pathogen appressorium to the plant surface (Howard et al., 1991). Cutin

monomers that contain one or more of the mid-chain hydroxyl group such as 10,16-dihydroxypalmitic acid and 9,10,18-tritrihydroxystearic acid are important for the activation of the cutinase gene in the pathogen (Podila et al., 1988). Although some pathogens can penetrate the cuticle through the secretion of enzymes, many of the biotrophic and hemibiotrophic pathogens such as *P. infestans* can penetrate the cuticle by the formation of an appressorium and a penetration peg (Kamoun and Smart, 2005).

2.5.1.1.3. Cellulose

Cellulose is a linear chain of D-glucopyranoses with β- (1→4) linkages and it is composed of 2000 to 6000 glucose residues. Sixty-seventy of the β- (1→4)-D-glucan chains are joined together by hydrogen bonds to form a microfibrill. The microfibrill are embedded in a mixture of hemicelluloses and lignin. Microfibrills are very important in the enforcement of the cell wall and they give the plant cell its shape (Carpita and McCann, 2000).

2.5.1.2. Cell Wall and Middle Lamella

The plant cell wall and the middle lamella are very important barriers against the pathogen infection. The cell wall is composed of different concentrations of pectic substances, cellulose, hemicelluloses, lignin, and proteins depending on the plant species and the growth stage. In addition, the primary cell wall contains appreciable amounts of proteins. The middle lamella is composed mainly of pectic substances in addition to lignin (Agrios, 2005).

2.5.1.2.1. Pectic substances

Pectic substances are grouped into acidic polysaccharides and neutral polysaccharides. The acidic pectic substances include: homogalacturonan, rhamnogalacturonan I (RGI), rhamnogalacturonan II (RGII). The neutral polysaccharides include: arabinan, galactan and arabinogalactan (Strasser and Amado, 2001). The RG I form the backbone for the attachment of other pectic substances and the hemicelluloses. *P. infestans* can hydrolyze pectic substances by producing two types of pectin methylesterase I and II. Methylesterase I, has a molecular weight of 45kD and an optimal pH of 7 while methylesterase II, has a molecular weigh of 35kD and an optimal pH of 6-8 (Forster and Rasched, 1985).

2.5.1.2.2. Hemicellulose

The hemicelluloses include a variety of substance. The most important polysaccharides from this group include: the homopolymers, xylans, β-1,3-glucan, β-1,6-glucan and the heteropolymers such as xylose, glucose, mannose, and galactose. In addition to their importance in the formation of the cell wall, the β- (1→3)-glucans are major component of callose that is formed during mechanical damages and pathogens attack (Huang, 2001).

2.5.1.2.3. Lignin

Lignin is an important component of the middle lamella and the secondary cell wall. It is a complex three-dimensional structure composed mainly of *p*-coumaryl, coniferyl and sinapyl alcohols which are synthesized via the phenylpropanoid pathway

(Douglas, 1996). It is extremely resistant against degradation and works as an effective barrier against many pathogens and gives a mechanical support to plant cells (Croteau et al., 2000).

2.5.1.2.3. Proteins

Proteins are also important components of cell wall. They combine with polysaccharides to form different groups of glycoproteins. The most important groups of the structural glycoproteins in the cell wall are: Hydroxyproline-rich glycoproteins (HRGPs), glycine-rich proteins (GRPs) and proline-rich proteins (PRPs) (Carpita and McCann, 2000). The most studied glycoproteins lectins, extensins and arabinogalactan belong to the HRGPs group. Lectins have the ability to agglutinate *N*-acetylglucosamine, a component of the cell wall of fungi. Extensins are basic glycoproteins and are structural component of the cell wall.

Cell wall-degrading enzymes and their products are important signal molecules that evoke plant defense responses and the accumulation of phytoalexins (Esquerre-Tugaye et al., 2000), pathogenesis related (PR) proteins (Palva et al., 1993), the enforcement of cell wall by lignification (Robertsen, 1987) and the accumulation of HRGP in the cell wall (Boudart et al., 1995).

2.5.2. Preformed Biochemical Defenses

In addition to structural defense mechanisms, many preformed compounds such as phenols, tannins, dienes, saponins, cyanogenic glycosides and glucosinolates are important in retarding pathogens (Reviewed by Cowan, 1999; Dixon, 2001;

Kliebenstein, 2004). Phenolic compounds are numerous in the plants and involved in the defense response against pathogens. The polymeric phenolic compounds i.e. flavonols, cinnamic acid derivatives and coumarins are more toxic than the phenolic monomers.

Osbourn (1996a) reviewed some other groups of the preformed antimicrobial compounds such as saponins, cyanogenic glycosides and glucosinolates. These compounds are commonly found in the outer cell layers of the plant tissues and they are usually stored in the vacuoles or other organelles in the healthy plants. Upon the damage of the plant organelles by an insect or a necrotroph, some of these compounds leak out and defend the plant against the attacking invader. Biotrophic fungi developed mechanisms to avoid damaging the plant cells in order to avoid these antimicrobial compounds (Mendgen and Hahn, 2002). Other mechanisms pathogens developed to escape these compounds include the detoxification or tolerating high concentrations of these compounds.

2.5.2.1. Saponins

Saponins are glycosylated compounds that belong to triterpenoid, steroid and steroidal glycoalkaloid groups (Osbourn, 1996b). The steroidal glycoalkaloids such as α-solanine and α-chaconine are abundantly found in the Solanaceae family including cultivated potato (Cowan, 1999; Bianco, 2002). Saponins toxicity is due to their ability to bind to sterols, located in the cell membranes of the pathogen, causing its perforation. Some pathogens such as *Pythium* and *Phytophthora* withstand saponins because of having a low concentration of sterols in their cell membrane (Arneson and Durbin,

1968). Other fungi, however, can detoxify saponins by the production of hydrolytic enzymes such as tomatinase (Bouarab, et al. 2002).

2.5.2.2. Cyanogenic glycosides

Cyanogenic compounds are found in many species. Cyanogenic compounds and their degrading enzymes are found in different compartments of the plant cell. Pathogens cause the tissue damage and hydrogen cyanide after a chain of reactions is produced. This gas inhibits metalloproteins such as cytochrome oxidase that is found in the mitochondria and inhibits respiration (Taiz and Zeiger, 2002). =

2.5.2.3. Glucosinolates

Glucosinolates are sulfur-containing compounds and are well known as mustard oil glycosides. They are found mainly in the plant family Brassicaceae that includes cabbage, broccoli, canola and radish. Like cyanogenic glycosides, glucosinolates and their hydrolytic enzymes are found in different compartments in the cell. Glucosinolates are also tissue specific (Kliebenstein, 2004). For example, different oilseed rape has been developed to maintain high levels of glucosinolates in their leaves and a low level of glucosinolates in their seeds (Taiz and Zeiger, 2002).

2.5.3. Induced Biochemical and Structural Defenses

2.5.3.1. Papillae formation

After the infection of potato leaves with *P. infestans*, drastic morphological alternations can be seen at the penetration site of the pathogen including cell polarization

and papillae formation. Papilla, a large brown dark plug, is composed of heterogeneous compounds and deposited between the plant plasma membrane and the cell wall (Schmelzer, 2002). Callose is a polysaccharide that is composed mainly of β-1, 3-glucan and it is the major component of papillae. Its synthesis in the plant can take place as early as 20 minutes after the pathogen attack. In addition to callose, silicon, thionine, peroxidase enzyme, phenolics, and hydroxyproline-rich glycoproteins (HRGPs) have been found in papillae. Silicon is believed to be involved in the activation of various enzymes during the pathogen attack (Schneider and Ullrich, 1994). Hydroxyproline-rich glycoproteins (HRGPs) increase the papillae physical strength and serve as a medium for the deposition of phenolic compounds and lignin. Furthermore, peroxidases help the deposition of some phenolic compounds in the papillae and enhance the defense response of the plant. Papilla is a non-porous structure and it reduces the fungitoxic compounds from entering the plant cell and prevents the uptake of food by the pathogen. Papilla formation can be activated by chitosan, fungal cell walls components, extracts from pathogen cell wall, and potassium phosphate (Huang, 2001).

2.5.3.2. Lignin and lignification

Lignification occurs during the infection by different pathogens. It enhances the plant defense response against pathogens. Deposition of lignin to the cell wall increases its mechanical strength and reduces its permeability (Croteau et al., 2000). Hydrogen peroxide H_2O_2 activates the oxidation of the phenolic compounds to quinones, which are more toxic, and the polymerization of lignin. During the hypersensitive response (HR),

higher levels of lignification have been reported. Oligo-β-glucans, glycoproteins, and chitin from the pathogen elicit the lignification of the cell wall.

2.5.3.3. Suberin and suberization

Suberin is a complex polymer composed mainly of long chain of fatty acids, alcohols, hydroxy fatty acids, and phenylpropane derivatives (Croteau et al., 2000; Taiz and Zeiger, 2002). It is formed during wound healing process and the formation of an abscission layer against some pathogens. It is deposited between the cell membrane and the cell wall. Suberin has two domains, the aliphatic domain that includes hydroxy fatty acids, esterified long chain fatty acids or dicarboxylic acid and the aromatic domain that is formed from caffeoyl, *ρ*-coumaryl, and coniferyl alcohols. Esterified glycerol plays a role in linking between the aromatic and the aliphatic domains (Moire et al., 1999). During the infection, peroxidase activity is found to be increased near the infected tissues and it catalyzes the formation of Suberin. Glucan, a cell wall component of *Phytophthora megasperma* f.sp. *glycinea,* elicits the production of Suberin in cotyledon tissues of soybean (Graham and Graham, 1991).

2.5.3.4. Tannins

In addition to their toxic effect on insects and animals, tannins have the ability to prevent infection by many fungi and bacteria. According to Taiz and Zeiger (2002), tannins are grouped into condensed and hydrolyzable tannins. The hydrolyzable group is a heterogeneous polymer that contains many phenolic compounds. The phenolic compound gallic acid is the most important constituent of this group. The condensed

tannins are created by the polymerization of many flavonoid subunits. Tannins have the ability to form a hydrogen bonds with the NH_2 and the SH groups of proteins. Tannins could be oxidized by phenol oxidase to produce tannins in the quinone form and that makes tannins more active and enables them to inactivate the pathogen's proteins by forming stable covalent bonds.

2.5.3.5. Cell wall proteins

The hydroxyproline-rich glycoproteins (HRGPs) are components of the cell wall. They contain hydroxyproline-rich lectins, extensins and arabinogalactan proteins. Accumulation of HRGPs is found to be higher in the resistant plants and occurs early in the incompatible host-pathogen interactions and late in the compatible interactions. HRGPs contain many basic amino acids and have the ability to agglutinate negatively charged cells and that may play a role in immobilizing bacteria (Leach et al., 1982). On the other hand, hydrogen peroxide in the early stage of the hypersensitive reaction mediates the insolubilization of the cell wall proteins. This adds mechanical strength to the cell wall (Brisson et al., 1994).

2.5.3.6. Phytoalexins

Phytoalexins are toxic antimicrobial metabolites produced by plants after the infection by pathogenic microorganisms or by chemical and mechanical injuries. They are mainly produced by dicotyledon crops and rarely by monocots and are mainly secondary metabolites. More than 300 chemicals of plant origin with phytoalexins properties have been reported (Huang, 2001). Phytoalexins are toxic to microorganisms,

animals and even to the plants themselves. In higher concentrations, they could adversely affect or threaten human life. Rosenkranz and Klopman (1990) predicted that 50% of phytoalexins have carcinogenic and mutagenic activities. The accumulation of phytoalexins in the infected plants could be a result of the direct activation of certain genes to produce specific enzymes or it could be a result of the stimulation of the inert forms of the phytoalexins by hydrolysis or the shifting in some metabolic pathways. Most of the phytotoxins interfere with the composition and the function of the membranes of the cell. They inhibit certain carriers in the electron transport system and repress the respiration in the mitochondria. To be effective in repressing a pathogen, the accumulation of phytoalexins in a certain plant should be high and fast. Different phytoalexins are produced by potato. Rishitin was first isolated in 1968 after the inoculation of the potato cultivar Rishiri with *P. infestans*. Other phytoalexins isolated from potato include: rishitinol, phytuberin, lubimin and solavetivone (Huang, 2001). In potato, these phytoalexins are tissue specific and are mainly produced in tubers but not leaves (Rohwer et al., 1987). Many pathogens evolved different ways to repress phytoalexins. Some pathogens have the ability to detoxify the phytoalexins by the production of enzymes. For example, the potato dry rot (*Fusarium sambucinum*= teleomorph: *Gibberella pulicaris)* has the ability to detoxify both lubimin and rishitin (Gardner et al., 1994). Elicitors can activate the phytoalexins production. They belong to different compound families including carbohydrates, peptides, glycoproteins, fatty acids or organic acid. For example, the polypeptide elicitins are produced by different species of *Phytophthora* and they have a molecular weight of about 10 KD. Cryptogein belong to this elicitins family and is produced by *P. cryptogea.* It activates the

accumulation of the phytoalexins capsidiol, phytuberol and phytuberin in the infected tobacco plants (Milat et al., 1991). In addition, arachidonic and eicosapentaenoic acids were found to elicit the production of the phytoalexins rishitin and lubimin in the infected potato tubers by *P. infestans* (Bostock et al, 1981). Glucans, cell wall components of *P. infestans*, were found to elicit the production of phytoalexins in potato tubers slices and the reduction of glucanase activities of the host (Andreu, et al., 1998). In addition, certain molecules such as hydrogen peroxides H_2O_2 and that are produced during the hypersensitive response could elicit the production of phytoalexins. For example, the water extract of the potato cultivar Rishiri treated with 1M H_2O_2 was found to elicit the production of the phytoalexin rishitin by potato (Monden et al., 1995).

According to Huang (2001), the major classes of phytoalexins are synthesized via the following pathways:

2.5.3.6.1. Phenylpropanoid pathway

In this process, the enzyme phenylalanine ammonia-lyase (PAL) converts phenylalanine to trans-cinnamic acid. The later is converted to a 4-coumaric acid (Huang, 2001; Taiz and Zeiger, 2002). Fritzemeier et al. (1987) found that the infection of potato leaves with *P. infestans* stimulates the overexpression of phenylalanine ammonia-lyase (PAL) and 4-coumarate CoA ligase (4CL) and this elevates the activity of the phenylpropanoid pathway. 4-coumaric acid undergoes different pathways and produces different phytoalexins. Examples include: Coumarins (i.e. Umbelliferone, Scoparone, and Xanthotoxin); Flavans (i.e. 7-Hydroxyflavan, 7,4'-Dihydroxyflavan and 7,4'-Dihydrox-8-methylyflavan); Flavanones (i.e. Betagarin, and Sakuranetin);

Anthocyanidins (i.e. Apegeninidin and Luteolinidin); Isoflavones (i.e. Daidzein, Formononetin, and Genistein); Isoflavnone (i.e. Kievitone, Vestitone); Pterocarpans (i.e. Medicarpin, Maackiain, Pisatin, Phaseollin and Glyceollins); Isoflavans (i.e. Sativan, Vestitol); Coumestans (i.e. Coumestrol, Psoralidin); others include: Magnolol, Eriobofuran, Malusfuran, Yurinelide, Irenolone, and Emenolone.

2.5.3.6.2. Acetic acid-mevalonic acid pathway

Phytoalexins from this group are produced via the mevalonate pathway wherein three molecules of Acetyl-CoA are joined together to form the 5-carbon isopentenyl diphosphate (IPP). The IPP joined with the 5-carbon dimethyallyl diphosphate (DMAPP), a product of the reaction between glyceraldehydes 3-phosphate and pyruvate through the methylerythritol pathway. The reaction produces the 10-carbon monoterpenes geranyl diphosphate (GPP). The GPP reacts with another IPP to form farnesyl diphosphate (FPP), a precursor used in the production of Sesquiterpenes (C15) and triterpenes (C30). Two GPP molecules can be connected to form geranylgeranyl diphosphate (GGPP), a precursor for the production of diterpenes (C20), tetraterpenes (C40) and the polyterpenes (Taiz and Zeiger, 2002). This pathway results in the production of many phytoalexins including: Monoterpenoid phytoalexins (i.e. Cupressotropolone A and B), Sesquiterpenoids (i.e. Solavetivone, Lubimin, Phytuberin, Phytuberol, Rishitin, Ipomeamarone, Capsidiol, Debeneyol, Glutinosone, Cichoralexin, lettucenin A, and many others), Diterpenoids (i.e. Casbene, Oryzalexins, momilactones, Phytocassanes A-E), Triterpenes (i.e. Uvaol, oleanol, ursolic acid and many others) (Reviewed by Huang, 2001).

2.5.3.6.3. Acetic acid-polymalonic acid pathway

In this pathway, Acetyl-CoA and Malonyl-CoA react in the presence of a fatty acid synthase enzyme to produce long-chain fatty acids. Those fatty acids undergo different modifications to produce polyacetylenes with triple carbon-carbon bonds that are the most common feature of the phytoalexins belonging to this group. This group includes: Falcarindiol, Falcarionl, Safynol. Acetyl-CoA combines with four molecules of Malonyl-CoA to produce the isocoumarin phytoalexin 6-Methoxymellin.

2.5.3.7. Pathogenesis-related proteins

Pathogenesis-related proteins (PR proteins) are a wide group of proteins that are produced by plants in response to pathogens attack. This group does not include the enzymes involved in the phytoalexins biosynthesis. The PR-proteins are mainly produced in incompatible host-pathogen interactions, but they could be also produced in compatible interactions. PR-proteins are extremely acidic or extremely basic. There are 14 families of PR-proteins (PR-1 to PR-14). The most studied PR-proteins belong to PR-2 family (β-1,3-glucanase) and PR-3 family (Chitinases). The PR-2 family are β-1,3-glucanase (33-45 KD). The potato plant cultivar Datura has been reported to produce β-1,3-glucanase and that has a molecular weight of 34,690 (Beerhues and Kombrink, 1994). In addition, Tonon et al. (2002) isolated an acidic 39 kD *β*-1,3-glucanase from potato cultivars having different degrees of field resistance to *P. infestans*. The levels of the *β*-1,3-glucanase were found to be higher in the resistant cultivars and it was speculated that this enzyme might have a major role in the field resistance. The PR-3

family produces different types of chitinases. Endo and exo- β-1,4-chitinases are important in the degradation of the chitin that is a cell wall component of many fungi. Chitin a linear homopolymers composed of β-1,4- linked N-acetyl-D-glucosamine. In potato plant, the activities of six of the chitinases were increased after the infection by *P. infestans* (Kombrink et al., 1988). This family includes five classes (I-V) characterized by the presence of different domains. Two of the class I isozymes have been identified in the potato cultivar Datura. The first one has a M_r of 31,119 with optimal pH of 6.8 and the second one has a M_r of 32,356 with optimal pH of 8.0 (Beerhues and Kombrink, 1994). Other important families include: the PR-4 family that produces chitosanases. They attack chitosan, polymer of β-1,4-D-glucosamine, the basic component of many fungi cell walls. The PR-5 family is called thaumatin-like PR proteins (TL proteins) and Thaumatin has five isomers. Treating potato plant with salicylate elicits the production of eight proteins. One of them belongs to TL proteins and has a molecular weight of 21kD (Pierpoint et al., 1990). In addition, infection of the potato plant by *P. infestans* activates the production of osmotin like proteins (Zhu et al., 1995). The PR-6 family includes protease inhibitors. They are small polypeptides with a range of 5-25 kD. The PR-9 family includes peroxidases that activate many reactions involved in the lignification, suberization and the incorporation of phenols to the cell walls of the plants.

2.5.3.8. Hypersensitive response (HR)

The hypersensitive response is a very powerful induced biochemical defense response that enables the plant to stop the pathogen infection in early stages. The HR can be seen clearly during the non-host resistance and is highly associated with race-

specific resistance. It is attributed to the presence of a receptor produced by a major (R) gene in the plant and that recognize a certain avirulent *(avr)* gene products of the pathogen, gene-for-gene interactions, (Heath, 2000; Christopher-Kozjan and Heath, 2003). Most often, the protein receptors are anchored to the inner or the outside of the plasma membrane or they could be found in the cytosol of the plant (Trewavas, 2000). In the resistant plants and in the early stages of the pathogen attack, the host nucleus moves towards the site of the pathogen infection. Dramatic changes in the actin cytoskeleton, nuclear size and appearance, cessation of the cytoplasmic streaming, and the breakdown of the protoplast can be clearly seen. Particles with Brownian motion inside the plant vacuole and the cleavage of the nuclear DNA become also clear. This is followed by the generation of hydrogen peroxide. The plasma membrane becomes impermeable and the protoplast starts to shrink and turn to brown as a result of the oxidation of the phenolic compound secreted from the adjacent cells. Granules of the nucleic acid inside the nucleus become more obvious and finally both the host cell and the pathogen are killed (Reviewed by Heath, 2000). In potato cells, it was reported that actin filament is involved in the activation of the HR (Furuse, 1999). The ability of the host to contain the pathogen depends on the speed of this HR. Vleeshouwers et al. (2000) reported the presence of the HR response in the interactions of *P. infestans* with some potato cultivars, wild *Solanum* cultivars and non-host plants. Results showed that in the non-host and the completely resistant wild *Solanum* spp.., the HR response was very fast and occurred within 22 h and result in the death of 1-3 cells. In contrast, in the partially resistant plants, the HR occurred between 16-46 h and resulted in the death of 5 or more cells. Some of the pathogen hyphae were able to escape the HR and initiate a

biotrophic interaction with the host. In addition, the accumulation of callose and the strengthening of the cell wall by phenolic compounds were reported in this study. Similar results were reported when the non-host plants such as parsley, *Solanum nigrum,* tobacco and *Arabidopsis* displayed HR after inoculation with *Phytophthora* spp. (Kamoun, 2001). The HR is also very important in the activation of the local and systemic acquired resistance (SAR) near the infection point of the pathogen infection and in the distal parts of the plant. That involved the increase in the production of the PR-proteins and the accumulations of phytoalexins (Kombrink and Schmelzer, 2001).

2.6. Signal transduction and disease resistance

Plant-pathogen interaction involves the activation of several genes in a plant. Signal molecules, specific receptors and secondary messengers are involved in this interaction.

2.6.1. Signal molecules

Signal molecules include elicitors, inducers and suppressors.

2.6.1.1. Elicitors

2.6.1.1.1. Exogenous elicitors

Many exogenous elicitors such as carbohydrates, proteins, and fatty acids can elicit the defense responses of the plant. Carbohydrate elicitors include glucans, chitin and chitosan. β-glucans are components of the fungal cell walls and are connected by β-(1→6) and β-(3→1) linkages. The smallest elicitor in this group is hepta- β-glucoside (Sharp et al., 1984). Chitin elicits the production of phytoalexins, PR-proteins and the

cell wall lignification in different plants. Chitosan elicits the production of callose and phytoalexins synthesis.

The protein family includes many elicitors such as α- and β-elicitins. Cato elicitin, capsicein, INF elicitin, cryptogeins and palmivorein are some examples of the elicitins that elicit the defense response in different plant species. The polypeptide elicitins that can be produced by different species of *Phytophthora* found to evoke hypersensitive response (HR) and systemic acquired resistance (SAR) in different *Nicotiana* species (Kamoun et al., 1993). In *P. infestans,* a major elicitin encoded by *inf1* gene, was found to be down regulated in the early stages of infection in the biotrophic phase, and to be highly expressed in the necrotic phase (Kamoun et al 1997). Glycoproteins were also found to elicit plant defense responses. For example, a 42 kD glycoprotein from *Phytophthora megasperma* f.sp. *glycinea* stimulates the H^+/Ca^+ influx, $K+^+/Cl^-$ efflux and the oxidative burst. This elicits the production of the phytoalexin furanocoumarin in parsley cells (Nurnberger et al., 1994).

Fatty acids located in the phospholipid membrane of many fungi could elicit the plant defense mechanisms. For example, arachidonic and eicosapentaenoic acids from *P. infestans* were found to elicit the production of the phytoalexins rishitin and lubimin in potato tubers (Bostock et al, 1981). Cerebrosides, a glycosphingolipid, and inositol sphingophospholipids have been also reported to work as elicitors.

2.6.1.1.2. Endogenous elicitors

This group contains a variety of compounds from the plant and that are produced as a result of the pathogens attack. Oligogalacturonides are released from the pectic

substances elicit the accumulation of the phytoalexins, lignification of the cell wall and PR-proteins production. The protein systemin elicit the plant to inhibit proteinases secreted by certain pathogens. In addition, phenolic compounds such as salicylic acid have been reported to signal and elicit the hypersensitive response, PR- protein plant production and the accumulation of phytoalexins.

2.6.1.2. Inducers

Inducers are signal chemical compounds secreted by plants during the compatible host-pathogen interactions. They elicit nodulation in rhizobium-legume symbiosis, tumor formation in crown gall diseases, the production of cell wall degrading enzymes and phytotoxins. For example, chemicals from the wax layer work as signals and stimulate the production of appressoria by some fungi. The phenolic compound acetosyringone and its derivatives, produced during mechanical injuries and wounds, work as a signal compound and activates the virulent gene (*vir*) expression in the crown gall bacteria *Agrobacterium tumifaciens* (Sheng and Citovsky, 1996).

2.6.1.3. Suppressors

Suppressors are compounds that are produced either by the pathogen or by the plant and prevent plant defense responses. Suppressors belong to a wide range of compounds including glycoproteins, oligosaccharides, toxins and many others. For example, glycan from *P. infestans* has been speculated to suppress the hypersensitive response in tomato plants (Storti et al., 1988). In potato cultivar Kennebec, different *P.*

infestans races were found to release the nonionic β-1,3-glucans that suppress the production of rishitin in the treated tubers (Doke et al., 1980).

2.6.2. Receptors

Receptors are located in different places in the plant cell such as the cell wall, the cytosol, the intracellular compartments and most of them are present in the plasma membrane (Trewavas, 2000). Recognition is the first step in the in host-pathogen interactions and is mediated by elicitors that must first bind to specific protein receptors in the host. This activates cascade of signaling events such as guanine nucleotide-binding proteins (G-proteins) activation, ion fluxes and the production of kinases. This leads to the biosynthesis of reactive oxygen species (ROS), jasmonic acid (JA), and ethylene and activates the defense genes of the plant. In addition, magnitude of the initial defense responses is fortified by the production of additional signal molecules such as lipid peroxidases, benzoic acid (BA) and salicylic acid (SA) (Hammond-Kosack and Jones, 1996).

2.6.3. Secondary Messengers

Secondary messengers are involved in the signal transduction pathway and they amplify and carry signals to effectors that activate the phenotypic expression. Secondary messengers include calcium ions, jasmonic acid, Inositol1,4,5-triphosphate (IP_3), Diacylglycerol (DAG), cyclic AMP, cyclic GMP, cyclic ADP-ribose (cADPR) and nitric oxide.

2.6.3.1. Calcium ion (Ca^{2+})

The Ca^{2+} concentration is kept at low levels inside the cytosol while it is found in a high concentration in the vacuole. The increase in the cytosol concentration of the Ca^{2+} ions in response to signals result in the activation of certain enzymes such as β-1,3-glucan synthase that activates the synthesis of callose and enhances the defense response of the plant. In addition, Ca^{2+} was reported to stimulate the arachidonic acid production and the accumulation of the phytoalexin rishitin in the infected potato plants (Zook et al., 1987).

2.6.3.2. Jasmonic acid and its derivatives

Phospholipids of the cell membrane are derivatives of many fatty acids such as palmitic acid (16:0), oleic (18:1), linoleic acid (18:2) and linolenic acid (18:3). Phospholipids are degraded by many phospholipases such as phospholipases A, B, C, and D. Lipoxygenase and other enzymes activate the conversion of linolenic acid to jasmonic acid (JA) and other derivatives (Liechti and Farmer, 2002). Nojiri et al. (1996) reported that SA is a signal compound that elicits the accumulation of the phytoalexin momilactone A in the rice plant. Moreover, Shulaev et al. (1997) found that methyl salicylate, a volatile liquid, work as an airborne signal and activates the defense responses of the healthy tissues of the infected and the neighboring plants.

2.6.3.2. Inositol phosphate and diacylglycerol

In the cell membrane, many phospholipids can be hydrolyzed by different phospholipases. For example, phosphatidylinositol (PI) is converted to PI 4-

monophosphat (PIP) and PI 4,5-bisphosphate (PIP2), PI 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) by certain enzymes. IP3 is then transferred to the cytosol where it mobilizes calcium from the vacuole, ER, the chloroplast and mitochondria. This results in the increase of Ca^{2+} in the cytosol and activates the production of many enzymes such as kinases, ATPases, lipases, calcium/calmodulin dependent enzymes and could elicit the production of phytoalexins. DAG also has an important role in the activation of protein kinases and phosphatases enzymes that causes protein phosphorylation or dephosphorylation and this triggers the nucleus gene expression (Trewavas, 2000). The increase in phosphoinositide has a positive role in the hypersensitive reaction (Atkinson et al., 1993). On the other hand, PIP2 could be hydrolyzed by phospholipase A (PLA) to produce unsaturated fatty acids that are attached by lipoxygenase and result in the production of JA.

2.6.3.3. Guanosine triphosphate (GTP)

During the signal transduction, guanine nucleotide-binding proteins (G-proteins) are involved. G-protein is a heterotrimeric that is located in the plasma membrane and projected towards the cytosol. This protein has three subunits α, β and γ. the GDP (Guanosine diphosphate) is attached to the γ subunit. When an elicitor or a ligand is attached to a receptor protein in the plasma membrane, it causes a conformational change in that protein and facilitates its attachment to the G-protein. Upon contact, GTP replaces the GDP of the α-subunit. This causes a conformational change in the α-subunit, causing it to dissociate from the complex, to bind and to activate another protein located in the plasma membrane known as adenylyl cyclase. This activation produces

cyclic adenosine monophosphate (cAMP) from ATP. The cAMP acts as a signal molecule and activates gene expression. After activation, the GTP is hydrolyzed to produce GDP and the α-subunit returned back to G-protein to make a new cycle (Trewavas, 2000; Huang 2001; Taiz and Zeiger, 2002).

2.6.3.4. Cyclic adenosine monophosphate (cAMP)

The cAMP is a secondary messenger and it elicits the production of the phytoalexin methoxymellein in carrot cells (Kurosaki et al., 1987). Other molecules for that involved in signal transduction include protein phosphorylation and protein dephosphorylation and oxidative burst. The reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide anion radical ($O_2^{\cdot -}$), and the hydroxyl radical ($HO^{\cdot}$) are highly reactive and very toxic. They are generated in the plants through many routes such as β-oxidation in glyoxysome, photosystem II in chloroplast, electron transport in mitochondria and other routes. The superoxide anion ($O_2^{\cdot -}$) is produced from O_2 by the help of NADPH oxidase. The ($O_2^{\cdot -}$) initiate the production of: 1) hydroperoxyl radical ($HO_2^{\cdot}$) and hydroxyl radical ($HO^{\cdot}$) that cause the lipid peroxidation in the plasma membrane and 2) hydrogen peroxide (H_2O_2) that causes the cross-linkage of the cell wall proteins and lignification. The superoxide dismutase (SOD) activates the conversion of $O_2^{\cdot -}$ to H_2O_2 . The later could produce the hydroxyl radical ($HO^{\cdot}$) or it could be detoxified by catalases or peroxidases enzymes to produce water (Hammond-Kosack and Jones, 1996 and 2000). ROS are involved in the production of the HR response, the oxidative cross-linkage of the cell wall proteins, and the signal transduction (Jackson and Taylor, 1996). In addition, they have been reported to elicit

the phytoalexins production in potato tubers (Ellis et al., 1993) and to cause the peroxidation of the plasma membrane phospholipids that activates the production of jasmonic acid.

2.7. Signaling and systemic acquired resistance (SAR)

In addition to the ability of the plant to resist pathogens at the site of infection, many plants have the ability to extend the resistance to distal parts of the plant and enhance the resistance to become active against different pathogens. This kind of resistance is known as SAR. Signal molecules are important in transmitting the message from the point of infection to the rest of the plant. Those signals elicit the plant to produce phytoalexins, PR-proteins and many other defense responses. Salicylic acid is a signal compound that has the ability to elicit SAR, which is usually accompanied by an increase in the concentration of H_2O_2 and is effective at distal parts of the plant (Chen et al., 1993; Jackson and Taylor, 1996). Other compounds that elicit SAR include 2,6-dichloroisonicotinic acid (DINA) and benzothiadiazole (BTH) (Vernooij et al., 1995 and Lawton et al., 1996).

PREFACE TO CHAPTER 3

Chapter 3 is comprised of a manuscript by myself, Dr. A. C. Kushalappa, Dr. W. D. Marshall, Dr. S. O. Prasher, and Dr. K. Al-Mughrabi. An abstract and a poster for this study have been presented in the "2nd Canadian Plant Genomics Workshop" held at Quebec City, August 2004. The paper has been submitted for publication in the Proceedings of the 3rd International Congress on plant Metabolomics held in Iowa, June 2004 and accepted for publication September, 2006. The contributions of the co-authors have been described in "Contributions to Authors" section. All literature cited has been placed at the end of the thesis.

Metabolic profiling, based on GC/MS, has been used to study the metabolites produced by soil-grown and in vitro-grown tubers (Roessner et al., 2000), in *Arabidopsis thaliana* mutants to discriminate metabolic phenotypes (Fiehn et al., 200b), to study a salt-stressed and non- stressed tomato plants, and to discriminate stems, leaves and roots of *Medicago truncatula* (Duran et al., 2003). This study reports the adaptation and standardization of metabolic profiling technology developed by Roessner et al. (2000) to metabolic profile potato cultivars susceptible and resistant to late blight. Two potato cultivars, susceptible cv. AC Novachip and moderately resistant cv. Caesar, were inoculated with *P. infestans.* The pathogen genotype US-8 was used here as it was more aggressive than US-1 genotype (Peters et al., 1999) and also more common in Canada (Medina et al., 1999; Peters et al., 2001; Daayf and Platt, 2003). Metabolites were analyzed using GC/MS.

Metabolic profiling is complex and a single plant species produces thousands of metabolites. There is no single solvent known to extract all the metabolites present in a plant. Different solvents such as methanol, methanol-water or ethanol have been used to

extract the polar metabolites from plant tissues (Julkunen-Titto, et al., 1996; Fiehn et al., 2000 a,b; Roessner et al., 2000; Jung et al., 2002). Slightly non-polar solvents such chloroform have also been used to extract the non-polar metabolites (Fiehn, 2000b). In this study, we used the polar extract of potato leaves and followed the protocol of extraction and derivatization procedures described by Roessner et al. (2000).

CHAPTER 3

Metabolic profiling of horizontal resistance in potato leaves (cvs. Caesar and AC Novachip) against *Phytophthora infestans*.

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3.1. Abstract

Metabolic profiles were developed for potato leaves, cvs. Caesar and AC Novachip, inoculated with water or *Phytophthora infestans*, using gas chromatography/mass spectrometry (GC/MS). The level of horizontal resistance was higher for cv. Caesar than for AC Novachip with an Area Under the Disease Progress Curve (AUDPC) of 334 and 857, lesion areas of 86 and 224 mm^2, and sporulation of 4.4 $x10^3$ and 13.7 x 10^3 sporangia $lesion^{-1}$, respectively. A total of 51 metabolites were consistently detected in all the four replicates of at least one treatment. Of the 51 relatively consistent metabolites, 33 were unique to a treatment and 18 were common to two or more treatments. A total of 7 and 29 pathogenesis related (PR) metabolites were identified in cvs. Caesar and AC Novachip, respectively. Most of the phenolic compounds were associated with the pathogen-inoculated AC Novachip. The metabolite heptadecanoic acid,was detected only in the pathogen-inoculated Caesar. In response to the *P. infestans* attack, the two cultivars appear to follow two different pathways. The susceptible cv. AC Novachip appears to follow the Shikimic acid-phenylpropanoid pathway as several phenolic metabolites and benzoic acids were detected, the latter being a precursor of the signal molecule salicylic acid (SA), known to trigger phenolic compounds. On the other hand, the moderately resistant cv. Caesar appears to follow mevalonic acid-methylerythritol pathway as heptadecanoic acid was detected, a probable derivative of linolenic acid that is a precursor of the signal molecule Jasmonic acid (JA), known to trigger terpenes. The factor analysis using principal components discriminated all the four treatments and the factor loading indicated which compound loaded

significantly to a treatment. The possible function of these compounds in plant defense against biotic stress is discussed.

Keywords: AC Novachip, Caesar, GC/MS, late blight, plant metabolomics, *Phytophthora infestans*, *Solanum tuberosum*, horizontal resistance.

3.2. Introduction

Potato late blight, *Phytophthora infestans* (Mont.) de Bary, is the most important pathogen that attacks potato (*Solanum tuberosum*) (Flier et al., 2003). *P. infestans* is a heterothallic pathogen requiring the A1 and the A2 mating types for sexual reproduction (Daayf and Platt, 1999; Peters et al., 1999; Stromberg et al., 2001). The A2 mating type was found to be more aggressive than the A1 (Fry and Smart, 1999). In Canada, the clonal lineage US-8 is the most aggressive and dominant on cultivated potato cultivars (Medina et al., 1999; Peters et al., 2001).

In the past, breeding programs have mainly considered vertical resistance, generally controlled by single or major R-genes, because transferring such genes from wild types to cultivated crops was relatively easy. Recently, many new races of *P. infestans* have been detected in North America. This has made the breeders to consider the horizontal resistance in the breeding programs, as it is considered to be more durable than the vertical resistance due to the polygenic nature of inheritance (Simmonds and Wastie, 1987; Peters et al., 1999). However, the progress made in transferring horizontal resistance to cultivated potatoes has been very limited because of the difficulty in breeding for polygenic traits (Evers et al., 2003).

In Plant-pathogen interactions, the complete pathway involves the binding of an elicitor, a suppressor or an inducer to a specific receptor, a messenger that carries the signal and an effector that activates the phenotypic expression. Pathogens produce different enzymes that can hydrolyze plant cell walls and their products act as signal molecules that evoke plant defense responses by the accumulation of phytoalexins (Esquerre-Tugaye et al., 2000), pathogenesis related (PR) proteins (Palva et al., 1993), the enforcement of cell walls by lignification (Robertsen, 1987) and the accumulation of hydroxyproline-rich glycoproteins (HRGP) in the cell wall (Boudart et al., 1995; Huang, 2001). Biochemical defense compounds produced by plants have been grouped into preformed phytoanticipins and induced phytoalexins that are synthesized following infection (Osbourn, 1996a). Some of the phytoanticipins commonly detected in plants include phenols, phenolic glycosides, sulfur compounds, saponins, cyanogenic glycosides and glucosinolates. Phytoanticipins are commonly found in the outer cell layers of the plant tissues and they are usually stored in the vacuoles or other organelles in the healthy plants. Following insect feeding damage or a necrotroph invasion, some of these compounds defend the plant against the attacking pathogen. Saponins are glycosylated compounds that belong to triterpenoid, steroid and steroidal glycoalkaloid groups. The steroidal glycoalkaloids are abundantly found in the Solanaceae family that includes potato. Some pathogens such as *Pythium* and *Phytophthora* withstand saponins because of low concentration of sterols in their cell membrane. Other phytoalexins isolated from potato include: rishitin, phytuberin, lubimin, solavetivone (Huang, 2001). Glucosinolates are sulfur-containing compounds and are well known as mustard oil glycosides.

Plant metabolites are numerous and they are estimated to be between 90,000 and 200,000 (Fiehn, 2001; Fiehn 2002). Primary metabolites are essential for the growth and development of the plant, while the secondary metabolites are not, and most of them are usually associated with the defense response in plant (Taiz and Zeiger, 2002). A given plant is considered to produce about 5000 secondary metabolites. Although the genomic, transcriptomic and proteomic information of plant-pathogen interactions is very useful in developing cultivars with novel traits, they do not always give sufficient information on the end products of plant defense, the metabolites produced in the host-pathogen interactions (Roessner et al., 2000; Fiehn 2001). Metabolic profiling has been used to identify genetically and environmentally modified traits (Roessner et al., 2001b). Roessner et al. (2000) were able to identify more than 150 metabolites from potato tubers using GC-MS technique. Major differences were found in the concentration of the amino acids such as glutamine, proline, and arginine. In vitro microtubers were found to have higher concentrations of the amino acids compared with the soil-grown tubers. Fiehn et al. (2000b) used metabolic fingerprinting for the comparison of two homozygous ecotypes and two single gene mutants of *Arabidopsis thaliana*. Distinct metabolic phenotypes were reported for each genotype.

Breeding for quantitative disease resistance is problematic due to lack of tools to evaluate quantitative resistance phenotypes. In segregating populations varying in low-to-high levels of quantitative resistance though the disease severity on very resistant and very susceptible plant-pathogen interactions were consistent among trials over years, those on the intermediate interactions were quite inconsistent (Haynes et al. 2002). Quantitative resistance in potato against late blight has been measured based on multiple

epidemiological disease parameters such as infection efficiency, latent period, lesion size, amount of sporulation, etc. (Carlisle et al., 2002). However, these measurements are time consuming and expensive for use in breeding programs. Plant breeders are looking for tools to measure phenotypes varying in quantitative resistance. Metabolic phenotyping of cultivars varying in resistance to disease may be a potential alternative. Accordingly, the main objective of this study was to develop metabolic profiles for potato cultivars with different levels of horizontal resistance against leaf infection by *P. infestans* and relate them to levels of resistance/disease severity.

3.3. Materials and methods

3.3.1. Potato plant production

Elite seed potato tubers of cvs. AC Novachip and Caesar were obtained from Bon Accord Elite Potato Center, NB and Global Agri. Services Inc., NB, respectively. AC Novachip is very susceptible and Caesar is moderately resistant to foliar infection by *P. Infestans* (CFIA, 2003). These tubers were stored at 4 °C and 90 %RH until use (Medina et al., 1999). Seed tubers were sown in individual16- cm- diameter pots containing soil mixture of 1:1 ratio of soil and PRO-Mix BX® (Premier Horticulture Ltd, Riviere-du-Loup, QC). Plants were fertilized weekly with 200 ml/pot of a solution (1.5g l^{-1}) of Plant-Prod® 20:20:20 and trace elements (Plant Products Co. Ltd., Ontario, Canada). Three stems per tuber were maintained. Potato plants were grown in the greenhouse (20-25 °C) for 30-40 days to obtain a good foliage growth.

3.3.2. Inoculum production

P. infestans culture (clonal lineage US-8, A2 mating type, isolate No. 1661, obtained from AAFC, Charlottetown, PEI) was maintained at 4 °C. The pathogen was sub-cultured on rye-agar seed extract media and incubated at 15 °C in darkness (Stromberg et al., 2001). The sporangia were harvested after 2-3 weeks by flooding with sterile water containing 0.02% Tween 80. The concentration of the sporangia in the suspension was adjusted to 5×10^4 $ml^{-1}$and the suspension was chilled at 5 °C for 2-3 hours to encourage the release of zoospores (Stromberg et al., 2001).

3.3.3. Inoculation and incubation

Plants grown in the greenhouse were transferred to a growth chamber and maintained at 20 °C, 16 h photoperiod and 90% relative humidity. Three days later, two plants were selected. One plant was inoculated by the pathogen and the second plant was inoculated by sterilized water and served as a control. On each plant, 6 well-formed leaflets were inoculated at both sides of midrib on the undersurface with 5 µl of the pathogen sporangial suspension containing 0.02% Tween 80 or by sterilized water containing 0.02% Tween 80 . Plants were misted with sterile water, covered with plastic bags to maintain high relative humidity, and transferred back to the growth chamber. The bags were removed 24 h later.

3.3.4. Disease Severity and Sporulation Assessment

The lesion diameter was measured on 1, 2, 4, 6, 8 days after inoculation (DAI) from which the lesion area and the Area Under the Disease Progress Curve (AUDPC) (Shaner and Finney, 1977) were calculated according to the following formula:

$$AUDPC = \sum_{i=1}^{n} [(x_{i+1} + x_i)/2] [t_{i+1} - t_i]$$

Where x_i = proportion tissue affected at the i *th* observation and t = time (days) after inoculation by the pathogen at the i *th* observation, and n = total number of observations.

Twenty-four hours after inoculation, leaf discs were cut using 15 mm cork borer, placed upper surface down in Petridishes lined with moist filter papers and incubated at 20 °C and 16 h photoperiod. The plates were completely randomized inside the incubator. Six days after inoculation, the leaf discs were transferred into a test tube containing 5 ml of 0.02% of Tween 80 in water, vortexed, and the number of sporangia was counted using a hemacytometer. Each sample was counted six times from which the average number of sporangia per lesion was derived.

3.3.5. Metabolite extraction and GC/MS analysis

Leaf discs containing the inoculated sites were cut at 24 h after inoculation, using a 15 mm cork borer and crushed in liquid nitrogen using a mortar and pestle. The powdered samples were stored in Eppendorf tubes at -80 °C until use. The polar metabolites were extracted following a method developed by Roessner et al. (2000) where 100 mg of the powdered plant tissue was extracted with 1.4 ml of 99.93 % methanol, for 15 min at 70 °C, vigorously mixed with one volume of water and centrifuged at 2,200*g* for 10 minutes. 1 ml of the methanol/water supernatant was dried in Speed Vacuum, methoximated in 80 µl of 20 mg of methoxyamine hydrochloride in pyridine for 90 minutes at 30 °C, and derivatized in 80 µl of N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) at 37 °C for 30 minutes. Ribitol (50 µl solution of 2 mg

ribitol ml^{-1} water) was added as an internal standard. 100 µl samples of leaf extracts, in tubes with septa, were loaded to auto sampler connected to GC (model 3400, Varian®, Canada) and 1µl samples were injected into the GC injection port. The GC was equipped with a capillary column (DB-5MS, 30 m and 0.25 mm diameter). The GC injector temperature was heated at 250 °C. Helium was used as a carrier gas with a flow rate of 1 ml s^{-1}. The oven temperature was held at 50 °C for 3 min and then increased at the rate of 3 °C min^{-1} until 200 °C, then at 12 °C min^{-1} until 250 °C and was held at this temperature for 2 min. The compounds were ionized and the mass spectra from 50 to 600 *m/z* were recorded using an ion trap analyzer. Data was analyzed using Saturn Lab Software, and NIST library was used to identify the metabolites. The data for the entire experiment were transferred into an EXCEL® (Microsoft Corporation) spread sheet, and sorted based on retention time using Pivot Table procedure. The mass spectra of individual peaks/compounds across four blocks of a treatment were manually compared among themselves using the retention time (±0.01 minute) as a reference, and with the top ten choices of NIST library search program. The output consisted of a list of compounds and their relative abundance of mass ions (ion trap detector output).

3.3.6. Experimental design

A completely randomized block design was used for the metabolic profiling study using GC/MS. The experiment consisted of two main factors of two cultivars of potato (Cesar and AC Novachip) and two sub-factors of inoculations (pathogen or water). The entire experiment was conducted four times over time. Each experimental unit for metabolic profiling consisted of 12 discs cut from 6 leaflets in 3 stems produced

from one tuber. The data on metabolites and their abundance were used in statistical analysis. From the same single-tuber-plants, used for GC/MS analysis, the lesions on another 6 leaflets were used for the assessment of disease severity over an 8-d period. The data on lesion diameter was used to calculate the lesion area and area under the lesion expansion curve. A completely randomized design with two treatments of two cultivars and four replicates was used for the sporulation assessment. Each experimental unit consisted of single-tuber-plants from which 10 leaflet-discs containing inoculation sites were cut. Spore suspensions were prepared 6 dai and the data on number of sporangia per inoculation site were used in statistical analysis.

3.3.7. Data processing and statistical analysis

3.3.7.1. Disease severity and sporulation

The data on average lesion area in mm^2, AUDPC, and the number of sporangia per inoculation site, for each experimental unit, were subjected to ANOVA using completely randomized design procedure of SAS.

3.3.7.2. Metabolic profile

The metabolic profiles consisted of GC/MS output on retention time, names and abundances of compounds. The data for the entire experiment were transferred into an EXCEL® (Microsoft Corporation) spreadsheet and the frequency of each metabolite occurrence among blocks was determined. The metabolites unique to a treatment (cultivars inoculated with water or pathogen) or metabolites common to two or more treatments were identified. The metabolites that are novel or that increased in abundance

following pathogen inoculation were designated here as Pathogenesis Related (PR) metabolites.

The compounds that were common to two or more treatments and their average mass ion abundances were normalized by dividing each by the total for all the metabolites considered and designated as metabolic fingerprints. The metabolic fingerprints were subjected to factor analysis (SAS), using the principle component method, to assign a compound or combinations of compounds that significantly loaded to a treatment and to discriminate resistance/treatments based on factor scores.

3.4. RESULTS

3.4.1. Disease severity and amount of sporulation:

The average lesion areas on the 8 DAI were 224 and 86 mm^2, and the average AUDPC were 857 and 334, for AC Novachip and Cesar, respectively. The amount of sporulation was higher in susceptible AC Novachip (13.7 $x10^3$ sporangia ml^{-1}) as compared to the moderately resistant cultivar Caesar (4.4 $x10^3$ sporangia ml^{-1}). An analysis of variance indicated that the treatments, from each of the three experiments, were highly significant at 1 % level and the F-values for the blocks, for lesion size and AUDPC, were not significant at 1% level.

3.4.2. Pathogenesis related (PR) metabolites and resistance level discrimination

A total of 875 metabolites were detected in the polar extracts of two potato cultivars, AC Novachip and Caesar, at 24 h after inoculation with *P. infestans* or water (control). Of these metabolites, 401 had mass ion abundances of $\geq$5000 (Ion Trap

detector output), including 51 metabolites that were detected in all the four blocks, in at least one of the treatments (Table 3.1). Out of the 51 metabolites, 33 were unique to a treatment, 12 were common to two or more treatments, and the remaining 6 were common to all the treatments (Caesar pathogen (CP), Caesar water (CW), AC Novachip pathogen (AP) and AC Novachip water (AW) inoculated). Two metabolites 1,4-Dihydro-2-isopropyl-6 and 2-Hydroxy-3-methylanthraquin were detected only in the CW. Another two metabolites 1H,10H-Furo[3',4':4a,5]napht and heptadecanoic acid, 16-methy were specific to CP. Thirteen metabolites were unique to AW and sixteen were unique to AP. A metabolite, 2-Methoxy-4'-nitro-diphenyla was detected in all the four blocks of AW, but occurred only in one block of CW. Two metabolites, 2-Phenyl-3-methoxy-cycloprop and 4H-1-Benzopyran-4-one, 5-hyd, were found in both cultivars when pathogen was inoculated, but the frequency and the abundances of these metabolites were much higher in the AP. In addition, Myo-inositol, 1,2,3,4,5,6-he and Acetic acid, (trimethylsilyl) were found in all the treatments, though their abundances varied. A total of 7 and 29 PR-metabolites were detected in Caesar and AC Novachip, respectively, including 3 that were common to both the cultivars (Table 3.1). These PR-metabolites can be used to discriminate resistance between these two cultivars.

3.4.3. Factor analysis to discriminate resistance levels

The metabolic fingerprints based on normalized abundances of 18 metabolites that were common to two or more treatments (excluding 31 metabolites unique to a treatment, out of 51 relatively consistent metabolites, Table 3.1) were subjected to factor analysis to discriminate resistance based on factor loadings. The factor scores were used

to discriminate resistance levels/treatments (Fig. 3.1). Nine compounds loaded significantly to factor 1 that contributed mainly to AP (metabolites number 1-9, Table 3.2), where five belonged to phenolic and alkaloid groups. Five metabolites (12-16, Table 3.2) loaded significantly to factor 2 which contributed mainly to AW, and one metabolite (18, Table 3.2) loaded significantly to factor 3 which contributed to CP.

3.4.4. Metabolite groups to discriminate resistance levels

The metabolites detected were further classified into different chemical groups based on their function, such as, phenolics, alkaloids, sulfur-containing, nitrogen-containing metabolites, and others (Table 3.1). The most frequent and abundant groups were the phenolics followed by the alkaloids. The phenolics were found in all the treatments with some differences in their abundances. Three phenolic metabolites were detected in both CW and CP, however, the abundances decreased following pathogen inoculation from 346 x 10^3 in CW to 220 x 10^3 in CP. On the contrary, the total number of the phenolic metabolites in the AP increased from 4 in AW to 9 in AP. The total abundances of phenolic compounds increased from 288 x 10^3 in AW to 322 x 10^3 in AP. The total abundances of benzoic acid derivatives increased from 56 x 10^3 in AW to 87 x 10^3 in AP. No benzoic acid derivatives were detected in either CP or CW. The total abundance of the phenolic metabolite 1,8-dihydroxyanthraquinone reduced in both the cvs. following pathogen inoculation, from 288 x 10^3 in CW to 188 x 10^3 in CP, and from 165 x 10^3 in AW to 83 x 10^3 in AP.

The number and abundances of alkaloids reduced following pathogen inoculation in both cultivars, however, both the number and abundances were higher for both AW

and AP. Similarly, the abundances of nitrogen-containing compounds reduced in pathogen inoculated treatments. Two sulfur-containing metabolites with total abundances of 44 x 10^3 in AW increased following pathogen inoculation to 272 x 10^3. No sulfur-containing metabolite was detected in both treatments of the cv. Caesar.

Table 3.1. Average abundance and frequencies of occurrence of metabolites (and PR-metabolites*) detected in leaves of two potato cultivars Caesar and AC Novachip inoculated with water or *P. infestans*.

Metabolites (Chemical groups) [1]	**CAS Number** [5]	**Mass ion abundance (x10^3)** [4]			
		CW	**CP**	**AW**	**AP**
Alkaloids		**69.4 [3]**[2]	**5.8 [1]**	**328.2 [9]**	**249.5 [5]**
1,4-Dihydro-2-isopropyl-6	70231-37-7	44.9 (4)[3]			
1H-Pyrimido[1,2-a]quinoline-	64399-32-2			66.0 (4)	
2,3-Dihydro-1H-2-methylcyclo	109682-72-6			36.7 (4)	
Conanin-3-amine, N,N-dimethy	14278-81-0			24.3 (4)	
Morphinan-14-ol, 8-azido-6,7	38211-23-3			73.4 (4)	
Pyrazolo[5,1-c]-as-triazine-	6726-58-5			35.1 (4)	
1,3-Dicyano-2-phenyl-3-aminocarbonyl-spiro(3,5)non-1-ene	NA				25.4 (4)*
Name: 1,2-Dihydro-2,4-diphenyl-quinazoline	NA			54.0 (4)	8.9 (2)
1,4-Dihydro-2-isopropyl-6-ph	70231-37-7			8.9 (2)	121.4 (4)*
Quinoline, 1,2-dihydro-1-(p-	13268-54-7	6.7 (1)		22.4 (4)	62.0 (4)*
Isoxazolo[4,3-a]phenazine, 1	21589-28-6	17.9 (2)	5.8 (1)	7.2 (2)	31.8 (4)*
Nitrogen		**12.4 [1]**		**99.1 [2]**	**48.5 [2]**
Methanamine, N-[4-(diphenylm	39129-62-9			39.8 (4)	

2,3-Di-O-benzoyl-d,l-glycero	84348-15-2				25.4 (4)*
Benzenesulfonamide, 4-(dimet	55670-22-9				23.2 (4)*
2-Methoxy-4'-nitro-diphenylacetylene	NA	12.4 (1)		59.3 (4)	
Phenolics		**345.8 [3]**	**219.6 [3]**	**288.1 [4]**	**321.7 [9]**
2-Hydroxy-3-methylanthraquin	91701-15-4	51.7 (4)			
Benzoic acid, 2,4-bis(trimethylsiloxy)-,	27798-55-6			55.6 (4)	
Noradrenaline tetraTMS	68595-65-3			51.3 (4)	
10-Dicyanomethylene-benz(a)anthrone	NA				23.3 (4)*
4H-1-Benzopyran-4-one, 6,7-d	24195-17-3				30.7 (4)*
9,10-Anthracenedione, 1-phen	1714-14-3				16.4 (4)*
Benzoic acid, 2,4-bis[(trimethylsilyl)oxy]-,	10586-16-0				64.3 (4)*
Benzoic acid, 3,5-bis(1,1-di	16225-26-6				22.4 (4)*
Luteoline (5,7,3',4'-tetrahy	491-70-3				22.1 (4)*
3-Hydroxy-4-(methylsulphonyl	80166-82-1	5.8 (1)			33.2 (4)*
9,10-Anthracenedione, 1,3-di	34155-88-9		23.8 (4)*	16.8 (3)	
4H-1-Benzopyran-4-one, 5-hyd	520-28-5		7.8 (1) *		26.8 (4)*
1,8-Dihydroxyanthraquinone d	7336-68-7	288.3 (4)	188.0 (4)	164.5 (3)	82.5 (3)
Sulfur				**43.8 [1]**	**271.5 [2]**
Tetramethyl diphosphan-oxide-sulfide	NA			43.8 (4)	

2-Methyl-2-(4-methoxyphenyl)-1,3-dithiane	NA				212.4 (4)*
exo-2-Cyano-endo-2-methylthiobicyclo[2.2.2]oct-5-ene	NA				59.1 (4)*
Others		**2478.4 [5]**	**2144.8 [10]**	**1897.3 [11]**	**2424.6 [14]**
1H,10H-Furo[3',4':4a,5]napht	65596-25-0		41.3 (4)*		
Heptadecanoic acid, 16-methy	5129-61-3		56.2 (4) *		
2-Ethoxycarbonyl-5-isopropyl-2-oxolene	NA			41.0 (4)	
3-(t-Butylacetoxy)-3-methylbutan-2-one	NA			9.3 (4)	
7,11-Dimethyloctadecane	65431-89-2			11.3 (4)	
Cyclopenta[c]pyran-1,3-dione	66407-26-9			29.5 (4)	
1,3,3-Trimethyl-6-hydroxy-2-	81968-80-1				30.5 (4)*
2,3,4,6-Tetramethoxystyrene	48153-74-8				30.7 (4)*
2-Propenoic acid, 3-(cyclohe	74367-01-4				40.1 (4)*
6-Benzyloxy-2-ethoxy-2,3,4,5	93939-03-8				50.0 (4)*
Octadecanoic acid, methyl ester	112-61-8				92.0 (4)*
2-Phenyl-3-methoxy-cyclopropane-1-carboxylic acid methyl ester	NA		8.5 (2)*		113.8 (4)*
Cyclopenta[c]pyran-4-carboxy	63785-74-0			26.9 (3)	106.8 (4)*
Hexadecanedioic acid diTMS	NA	33.4 (2)	33.3 (1)		65.8 (4)*
3'-Methyl-2-benzylidene-coumaran-3-one	NA		37.6 (3)*	63.9 (4)	61.4 (4)

Tricyclo[3.2.1.02,4]octane,	66930-02-7		11.0 (2)*	20.2 (4)	58.5 (4)*
(3R,6R)-(+)-3-Isopropenyl-6-methyl-cycloheptanone	NA	159.6 (4)	99.7 (2)	97.5 (2)	102.5 (2)*
Acetic acid, [(trimethylsily	33581-77-0	1246.7 (4)	1123.4 (4)	1166.4 (4)	1291.5 (4)*
Myo-Inositol, 1,2,3,4,5,6-he	2582-79-8	907.3 (4)	633.5 (4)	148.2 (4)	172.7 (4)*
Propanoic acid, 3-(trimethyl	18296-04-3	131.4 (4)	69.6 (2)	161.7 (4)	192.8 (4)*

[1] Shortened chemical names according to NIST Library.

[2] Total numbers of metabolites per group for the corresponding treatment.

[3] Frequency of metabolites among four blocks; * = Pathogenesis Related (PR) metabolites (abundances of CP>CW; AP>AW).

[4] the total abundance of metabolites ≥5000 (Ion Trap Detector output) were only include.

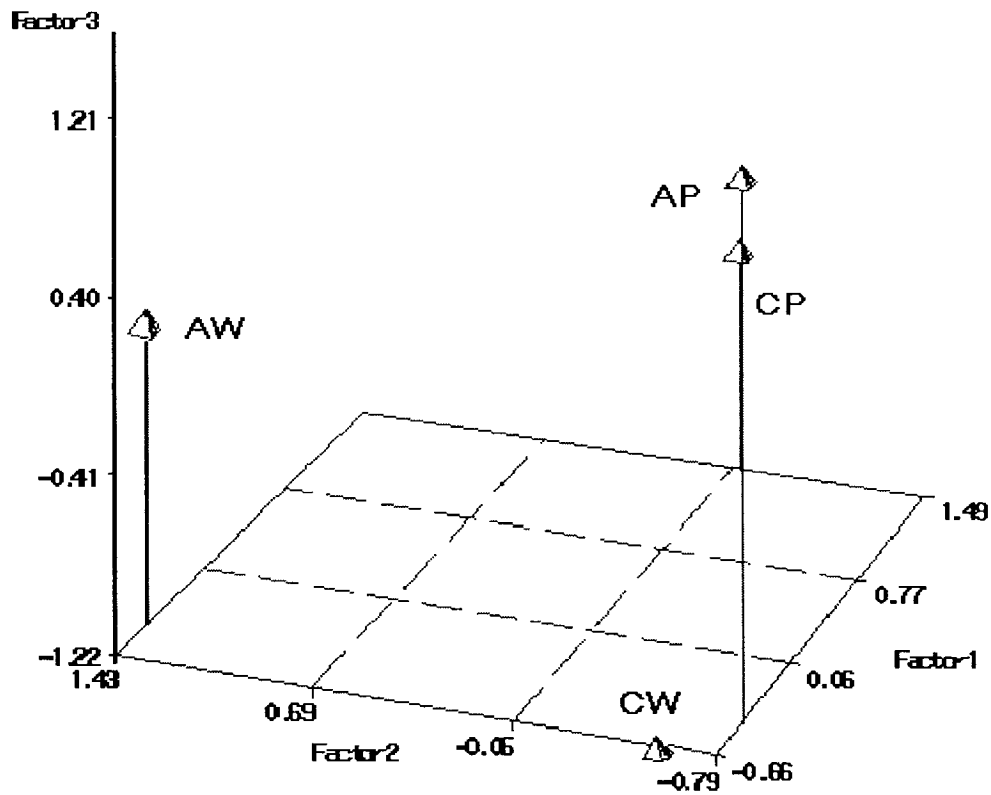
[5] CAS Registry Number = Chemical Abstract Service Registry Number; NA= Not Applicable. Whenever the CAS registry number has been found, the complete name of the metabolite is included.

Table 3.2. Eigenvector loadings[1], based on factor analysis using principal component method, for normalized abundances of 18 metabolites that were common to two or more treatments (metabolites unique to treatments not included; details in Table 3.1)

No.	Metabolites	Factor 1	Factor 2	Factor 3
1	2-Phenyl-3-methoxy-cycloprop	0.994[1]	-0.094	-0.039
2	1,4-Dihydro-2-isopropyl-6-ph	0.994	0.041	-0.099
3	3-Hydroxy-4-(methylsulphonyl	0.962	-0.112	-0.245
4	Cyclopenta[c]pyran-4-carboxy	0.960	0.269	-0.075
5	4H-1-Benzopyran-4-one, 5-hyd	0.957	-0.233	0.167
6	Tricyclo[3.2.1.02,4]octane,	0.946	0.295	0.130
7	Quinoline, 1,2-dihydro-1-(p	0.910	0.385	-0.149
8	Isoxazolo[4,3-a]phenazine, 1	0.887	-0.106	-0.447
9	Hexadecanedioic acid diTMS	0.772	-0.635	-0.022
10	(3R,6R)-(+)-3-Isopropenyl-6-	-0.752	-0.059	-0.656
11	1,8-Dihydroxyanthraquinone d	-0.979	-0.116	-0.167
12	1,2-Dihydro-2,4-diphenyl-qui	-0.153	0.982	0.102
13	2-Methoxy-4'-nitro-diphenyla	-0.358	0.933	-0.009
14	Acetic acid, [(trimethylsily	0.116	0.861	0.493
15	Propanoic acid, 3-(trimethyl	0.512	0.819	-0.255
16	3'-Methyl-2-benzylidene-coum	0.394	0.747	0.535
17	Myo-Inositol, 1,2,3,4,5,6-he	-0.639	-0.758	-0.123
18	9,10-Anthracenedione, 1,3-di	-0.470	0.214	0.855

[1] Eigenvector (metabolite) loadings with high positive values indicate significant loading of compounds to each factor. Factor 1 contributed mainly to AC Novachip-pathogen-inoculated, Factor 2 = AC Novachip-water-inoculated; factor 3=Caesar-pathogen-inoculated (see Fig. 3.1 for factor scores). Eigenvectors are linear combination of the original variables and they measure the strength of the relationship between the original variables and the hidden or latent variables.

Figure 3.1. Scatter plot (using factor scores) of potato cultivars with contrasting levels of horizontal resistance, pathogen or water inoculated, based on factor analysis of normalized abundance of 18 compounds common to two or more treatments. AW, AP = water or pathogen inoculated AC Novachip and CW, CP = water or pathogen inoculated Caesar, respectively.


Factor3
1.21
0.40
-0.41
-1.22
AP
CP
AW
CW
1.49
0.77
0.06
-0.66
Factor1
1.43
0.69
-0.05
-0.79
Factor2

3.5. Discussion

In the present study, we were able to discriminate levels of resistance in two potato cultivars inoculated with *P. infestans* based on metabolic profiling of the polar portion of the plant extract using several criteria: a) unique and combinations of PR-metabolites; b) chemical groups of metabolites and their abundances; and c) factor models based on normalized abundances of metabolites common to two or more treatments. The cv. AC Novachip produced more PR-metabolites, including phenolic compounds than cv. Caesar. The cv. Caesar produced high abundance of heptadecanoic acid, 16-methyl following pathogen inoculation. The total abundances of phenolics were higher for AP than for CP. Also, the factor loadings and scores, based on common metabolites, discriminated the resistance levels.

The cv. Caesar was more resistant than cv. AC Novachip for leaf infection by *P. infestans* as confirmed by the lower disease severity (lesion area and AUDPC) and lower amount of sporulation. This is in agreement with the assessment of resistance previously reported by CFIA (2003).

Many metabolites detected in our study were identified as intermediate compounds of metabolic pathways of plant-pathogen interaction. Based on the compounds detected in this study, it appears that the two cultivars follow two different metabolic pathways to defend against *P. infestans* attack. The cv. AC Novachip appears to follow shikimic acid pathway producing more of phenolics, while the cv. Caesar appears to follow mevalonic-acid pathway producing terpenoids, Heptadecanoic acid. Majority of the compounds detected in this study belonged to the phenolics. This group

contains many metabolites that are known for their antimicrobial activity (Dixon et al., 2002). Phenolic compounds are produced via Shikimic acid-phenylpropanoid pathway where the amino acid phenylalanine is converted to trans-cinnamic acid that in turn produces many metabolites belonging to different sub-groups such as coumarins, flavones, flavanones, flavonols, isoflavans, isoflavones, anthocyanidins and many others (Dixon et al., 2002; Huang, 2001; Hopkins and Huner, 2004). The shikimic acid pathway also can increase the production of the aromatic amino acids phenylalanine, tyrosine and tryptophan (Dixon, 2001; Taiz and Zeiger, 2002; Lyon, 2003).

The activity of the phenolic compounds, total numbers and abundances, was higher in the cv. AC Novachip than the cv. Caesar. The total abundance of benzoic acid derivatives increased from 56 x 10^3 in the AW to 87 x 10^3 in the AP, while these were not detected in Caesar. Benzoic acid was reported to be a precursor of the signal molecule salicylic acid (Dixon et al., 2002; Meraux, 2002; Nakane et al., 2003; Lyon, 2003). Although the SA was not detected in our study the presence of the benzoic acid derivatives that are precursors of SA may indicate its activity. Following a pathogen attack, plants produce SA, which in turn stimulates the production of PR-proteins (Durner et al., 1997) and phytoalexins (Nojiri et al. 1996) leading to both local (LAR) and systemic acquired resistance (SAR) (Heil and Bostock, 2002). The abundance of the phenolic metabolite 1,8-dihydroxyanthraquinone was reduced by about 35% in Caesar and 50% in AC Novachip following pathogen inoculation. This metabolite is an anthraquinone and could have been produced from the precursor isochorismic acid (Lyon, 2003). The reduction in anthraquinone production can stimulate the production of salicylic acid with the help of the enzyme pyruvate lyase. This pathway is proposed in

Arabidopsis, but not yet proven in potato (Lyon, 2003). If this is true, then one could expect that the amount of salicylic acid produced via this pathway would be more in the AP than in the CP.

Although the activity of nitrogen-containing compounds and alkaloids were reduced in both cultivars when inoculated with the pathogen, the reduction was more in the cv. Caesar. The primary sources of these compounds are pyruvate, the tricarboxylic acid cycle (TCA) and the Shikimic acid pathway (Taiz and Zeiger, 2002; Nakane, 2003). Thus, we could hypothesize that either the activity of pyruvate, the TCA, or the activity of one branch of the shikimic acid pathway that produces aromatic amino acids was reduced.

Other metabolites which have phenol and nitrogen in their structures and might be synthesized via shikimic acid-phenylpropanoid pathway by AC Novachip were: (9,10-Anthracenedione, 1,3-di; 9,10-Anthracenedione, 1-phen; 2,3-Di-O-benzoyl-d,l-glycero; Benzenesulfonamide, 4-(dimet; Luteoline (5,7,3',4'-tetrahy; 10-Dicyanomethylene-benz(a)a; 4H-1-Benzopyran-4-one, 6,7-d). Factor analysis (Table 3.2) indicated significant loading of nine metabolites to factor 1 and these were significantly correlated with the pathogen-inoculated AC Novachip. Five of these metabolites belong to phenolics and alkaloids.

In pathogen-inoculated Caesar Heptadecanoic acid was detected which is a fatty acid that could be a derivative of heptadecatrienoic acid (C17: 3) that is produced from linolenic acid (C18: 3) through the activity of α-dioxygenase, a pathogen inducible oxygenase (Lyon, 2003). In response to pathogen attack, it has been reported that in the mevalonic acid pathway lipoxygenase and other enzymes activate the conversion of

linolenic acid to jasmonic acid (JA) and other derivatives (Liechti and Farmer, 2002). Although the plants can produce JA following insect attack or the infection by a pathogen, the end products of these interactions are different, and these affect the products of the local and systemic acquired resistance in the plant (Fidantsef et al., 1999). In the Solanaceae, the jasmonates and oxylipins (linolenic acid derivative) elicit proteinase inhibitor accumulation and steroid glycoalkaloid synthesis responses, i.e. similar to mechanical wounds or chewing insect attack (Choi et al., 1994; Casey, 1995). On the contrary, fungal elicitors such as arachidonic acid (AA) and lipoxygenase metabolites stimulate the accumulation of PR-proteins and phytoalexins, leading to programmed cell death (Bostock et al., 1986). Because high abundance of 'Heptadecanoic acid, 16-methy' was detected in cv. Caesar, it could be hypothesized that the mevalonic-acid pathway was active, which is signaled by JA. However, no JA and little lipophilic metabolites were detected in our study, but in reality, there may have been more as in the present investigation only polar phase of the plant extracts (methanol-water solvents), which excluded most lipophilic compounds including many terpenes, was analyzed.

P. infestans is a hemibiotroph and during the biotrophic phase, the pathogen usually shows minimal secretary activity in order not to trigger the plant defense responses (Mendgen and Hahn, 2002). It has been found that the pathogen elicits the hypersensitive response (HR) when infecting non-host, completely and partially resistant plants. In the non-hosts plants and the completely resistant *Solanum* species, the HR was very fast and killed 1-3 infected plant cells (Vleeshouwers et al., 2000). However, in the partially resistant plants, the HR was slow and gradually killed five or more plant cells.

The major R-genes such as R1 were found to produce a strong HR response while the weak R-genes i.e. R10 was found to produce a weak and a slow HR response and the pathogen hyphae were able to grow beyond the HR lesion and were able to start new infections (Vleeshouwers et al., 2000). In our study, both cultivars are believed to have minor genes because they were found to have different levels of disease severity according to the results of the lesion size, sporulation rates and the AUDPC. Therefore, both cultivars must have evolved different strategies, other than the HR, to postponed infection by *P. infestans* and this result in the activation of different metabolic pathways.

In conclusion, the cultivars tested in this study, AC Novachip and Caesar seem to have evolved developing two separate mechanisms to suppress the invasion by *P. infestans*. The activity of phenolic metabolites increased in AC Novachip, in both numbers and abundances, which are known to be produced through the shikimic acid pathway. On the other hand, heptadecanoic acid, a potential precursor for the signal molecule jasmonic acid, was only detected in the inoculated Caesar. JA has been associated with the activation of many terpenes. An analysis of the lipophilic portion of the plant extract would shed more lights on the compounds that are involved in this pathosystem.

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PRFACE TO CHAPTER 4

Chapter 4 is comprised of a manuscript by myself, Dr. A. C. Kushalappa, Dr. W. D. Marshall, Dr. K. Al-Mughrabi, and Ms. A. Murphy. The manuscript was submitted with revisions to "European Journal of Plant Pathology" in March 2006 in August 2006. In addition, an abstract and a poster of this chapter have been presented in the "4th International Conference on Plant Metabolomics" held at Reading Berkshire UK, April 2006. The contributions of the co-authors have been described in "Contributions to Authors" section. All literature cited has been placed at the end of the thesis.

In the previous study, we had some problems especially in the consistency of the metabolites among the 4 replicates (blocks) and that was attributed to several factors. For example, the potato plants were grown in the greenhouse and 4 replicates were conducted over time. As a result, the biological variations among the 4 replicates could have been high due to the relative variations in the temperature and the light intensity. In this study plants were grown in a growth bench under controlled conditions including temperature and light intensity. On the other hand, we had problems with the metabolites identification and quantification. The total abundances of several metabolites were very low among the 4 replicates and part of the variation was attributed to the injection of low concentrated samples into the GC/MS and to the high bleeding of the GC column that masked the detection of many metabolites. According to the NIST library list of the top 10 suggested names, the metabolite with the highest probability was selected and considered as a tentatively identified metabolite. Afterward we discovered that this assumption was not always correct. In this study, 5 replications instead of 4 were used and both polar and non-polar metabolites were analyzed to increase the range of the detected metabolites. Improved protocols developed by Fiehn et al., (2000a, b) were used and adopted later for

developing/standardizing a protocol for metabolic profiling of potato-late blight interaction metabolites. In addition to the internal standards, three retention time index standards (Chrysene, Naphthalene, and Phenanthrene) were added to the extracted samples before injection into GC/MS as suggested by Dr. William Marshal to calculate the Lee Retention Index (RI) for each of the reported metabolites. According to Eckel (2000), metabolites with boiling points less than their RI were considered as incorrectly identified by the NIST library. To improve the accuracy of the metabolites identification, the GOLM metabolome database (Kopka et al., 2005) was used to confirm the names suggested by the NIST library for all the reported metabolites.

This study reported the effect of incubation time on the expressed metabolites after the pathogen inoculation and results were used to select the optimum incubation time for the next study. Because of volume involved in this study only one cv. AC Novachip was profiled. In this study, a comprehensive statistical approach was used for data analysis. Both ANOVA and FACTOR analysis, respectively, were used to classify Pathogenesis Related metabolites (PR-metabolites) and to elucidate the biological functions and plausible pathways associated with these metabolites. Results showed a dynamic change in the abundances of many of the expressed metabolites in response to pathogen attack. Most of the metabolites expressed in the first and second day after inoculation belonged to organic and amino acids. In the second day after pathogen inoculation, the amino acids phenylalanine and tyrosine were found to be highly increased in abundances. These metabolites are potential precursors for the phenylpropanoid pathway that produces many of the plant defense secondary metabolites, as it will be discussed later.

CHAPTER 4

Temporal dynamics of pathogenesis related metabolites and their plausible pathways of induction in potato leaves following inoculation with *Phytophthora infestans*.

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4.1. Abstract

Metabolite profiles based on GC/MS were used to study the temporal dynamics of metabolites in potato leaves following pathogen inoculation. In the polar and the non-polar plant extracts a total of 106 consistent peaks were detected, of which 95 metabolites were tentatively identified. Following pathogen inoculation the abundances of 42 metabolites were significantly increased or decreased, and these metabolites were designated as Pathogenesis Related (PR)-Metabolites. Factor analysis of abundances of 106 metabolites identified four plant-pathogen interaction functions: i) Homeostasis; ii) Primary defense; iii) Secondary defense; iv) Collapse of defense responses. During the primary and secondary defense phases, dramatic changes in the amino acids, known precursors of several plant defense related metabolites, were observed. The plausible satellite-networks of metabolic pathways leading to the up-regulation of these families of amino acids and other secondary metabolites, and their potential application for the evaluation of horizontal resistance in potato against late blight pathogen is discussed.

Abbreviations: GC/MS = gas chromatography/mass spectrometry; PR-metabolites = pathogenesis related metabolites, increased (PRI) or decreased (PRD) in abundances.

Key words: Metabolomics, GC/MS, *Solanum tuberosum*, horizontal resistance, factor analysis, Lee retention index, PR-metabolites.

4.2. Introduction

Late blight, caused by *Phytophthora infestans* (Mont.) de Bary, is one of the most destructive diseases of potato (Flier et al., 2003). *P. infestans* is a heterothallic

pathogen that requires both A1 and the A2 mating types for sexual reproduction (Daayf and Platt, 1999; Peters et al., 1999; Stromberg et al., 2001). In Canada, the clonal lineage US-8 (A2 mating type) is the most aggressive and dominant on cultivated potato cultivars (Medina et al., 1999; Peters et al., 2001). Systemic fungicides are used extensively to manage this disease, which have lead to the development of resistant populations. Accordingly, the use of resistant cultivars is considered to be the best option to manage this disease.

Breeding for vertical resistance is easier than horizontal resistance. At least 11 vertical resistance R genes against *P. infestans* have been introduced into cultivated potato from the wild potato *Solanum demissum* (Wastie, 1991; Gebhardt and Valkonen, 2001). Unfortunately, vertical resistance is not as durable as the horizontal resistance. Thus, the later is the current focus of many breeding programs (Simmonds and Wastie, 1987; Peters et al., 1999). However, breeding for horizontal resistance in potato is difficult because it is controlled by several genes (Evers et al., 2003). The quantitative resistance in potato against late blight is generally measured based on multiple epidemiological disease parameters such as the latent period, the lesion size, and amount of sporulation (Carlisle et al., 2002). However, these techniques are not only time-consuming but also don't explain the mechanism of resistance. Metabolite profiling is an alternative tool for breeders to do high throughput phenotyping of resistance.

Plants defend abiotic and biotic stresses through the development of structural barriers and production of chemical compounds such as signal molecules and phytoalexins (Osbourn, 1996a; Kombrink and Schmelzer, 2001; Montesano, 2003). Following pathogen invasion, plants produce pathogenesis related proteins (PR-proteins)

(Palva et al., 1993) and pathogenesis related metabolites (PR-metabolites) (Hamzehzarghani et al. 2005). Even though the plant genes, transcripts and proteins are expressed following pathogen attacks they do not provide sufficient information on the end products of genes (metabolic phenotypes). Therefore, metabolomics should be included as an integral part of functional genomics, for a comprehensive understanding of the plant-pathogen interaction (Fiehn, 2001; Sumner et al., 2003; Bino et al., 2004; German et al., 2005). Accordingly, metabolite profiling has been used to discriminate genetically modified traits (Roessner et al., 2001b; Mungur et al., 2005), growth of potato microtubers in-vitro and in soil (Roessner et al., 2000), homozygous ecotypes from single gene mutants in *Arabidopsis thaliana* (Fiehn et al., 2000b), wheat cultivars varying in resistance against disease (Hamzehzarghani et al., 2005), *Medicago truncatula* exposed to biotic and abiotic stress factors (Broeckling et al, 2005), and salt-stressed tomatoes from the non-stressed ones (Johnson et al., 2003).

P. infestans is a hemibiotroph, where in a compatible plant-pathogen interaction, the pathogen begins its invasion as a biotroph penetrating epidermal layers in about 16 hours and producing haustoria in about 22 h. Around 46 h, the pathogen becomes a necrotroph establishing well in the host and producing sporangia (Vleeshouwers et al., 2000). So, it would be very interesting to see the temporal dynamics of metabolites following pathogen invasion, and such a study can lead to a better understanding of plant-pathogen interactions. The objectives of this study were to use metabolomics to study the temporal dynamics of metabolites and their plausible pathways of production with the disease development following inoculation with *P. infestans*.

4.3. Materials and methods

4.3.1. Potato plant production

Elite seed tubers of the potato cultivar AC Novachip were obtained from the Potato Research Center, Agriculture and Agri Food Canada, New Brunswick. Tubers were planted in 16 cm diameter pots (one tuber per pot) containing mixture of 1:1 ratio of soil and PRO-Mix BX® (Premier Horticulture Ltd, Riviere-du-Loup, QC) and maintained at 20 °C, 16 h photoperiod and around 70% relative humidity in a growth bench. Plants were fertilized weekly with 200 ml pot^{-1} of a solution (1.5g per L) of Plant-Prod® 20:20:20 containing trace elements (Plant Products Co. Ltd., Ontario, Canada). One to three stems per plant/pot were maintained.

4.3.2. Pathogen

P. infestans (clonal lineage US-8, A2 mating type, isolate (1661) was obtained from AAFC, Charlottetown, PEI. The pathogen was sub-cultured on Rye-Agar media and incubated at 15 °C (Stromberg et al., 2001). After 2-3 weeks a sporangial suspension was made with sterilized water containing 0.02% Tween 80. The concentration of spores in the suspension was adjusted to 1.0×10^5 sporangia ml^{-1} and the suspension was chilled at 5 °C for 2-3 hours to encourage the release of zoospores (Stromberg et al., 2001).

4.3.3. Inoculation and incubation

Three days before inoculation, 5-6 week old plants grown in growth benches were transferred to a growth chamber maintained at 20 °C, 16 h photoperiod and 90%

relative humidity. Six completely developed leaflets, from 1-3 stems of a single-tuber-plant, were inoculated at two sites, once on each side of the midrib, with 5 µl of the sporangial suspension. The plants were misted with sterile water, covered with plastic bags to maintain high relative humidity, and transferred back to the growth chamber. The bags were removed 24 h after inoculation.

4.3.4. Disease Severity and Sporulation Assessment

The diameter of lesions was measured at 2, 4, 6, and 8 d after inoculation. The largest and the smallest diameters of each lesion were first measured and then divided by 4 to obtain the average radius of each lesion. The area was calculated then by using the formula: Area = πR^2, where R is the average radius of a lesion.. For the sporulation assessments, 24 h after inoculation leaf discs with *P. infestans*, sites were cut using 18 mm diameter cork borer. Four replicates of ten discs per a Petridish were used. The Petridishes lined with moist sterile filter papers were incubated at 20 °C and 16 h photoperiod. After 6 d, discs were transferred to a test tube containing 3 ml aqueous solution of 0.02% Tween 80 and vortexed. The number of sporangia was determined using a hemacytometer and represented as number of sporangia per leaf disc.

4.3.5. Metabolite extraction and GC/MS analysis

Leaf discs containing the inoculation sites were cut using 15 mm cork borer, treated with liquid nitrogen and stored at -80 °C for a few days, lyophilized for 48 h and returned to the storage at –80 °C until extraction. The polar and non-polar metabolites were extracted following methods developed by Fiehn et al. (2000a,b) with minor

modifications. The lyophilized tissue was first crushed in liquid nitrogen and the polar metabolites were extracted from a 30 mg sample using 1.4 ml of methanol and 50 μL of water. To the same sample 50 μL of ribitol (0.2 mg ml^{-1} of water) and 50 μL of nonadecanoic acid methyl ester (2 mg ml^{-1} of chloroform) were added as internal standards. The samples were heated at 70 °C for 15 min. and centrifuged at 13,500 rpm for 3 min. The supernatant was transferred to a glass tube with a screw cap with teflonized inlays. 1.4 ml of pure water was added. For the non-polar pellets, 0.75 of chloroform was added, vortexed and heated at 37 °C with continuous shaking for 5 minutes. Samples were centrifuged at 13,500 rpm for 3 min. The non-polar supernatant and the previously obtained water/methanol extract were mixed together, vortexed and centrifuged at 3,800 rpm for 15 min. The upper polar phase was decanted into another tube and filtered through 15 ml Millipore tubes. One ml of this extract was dried using a Speed Vac., and 50 μL of methoxyamine hydrochloride (20 mg ml^{-1} pyridine) was added to the dried sample. The samples were heated with continuous shaking at 30 °C for 90 min and derivatized by adding 80 μL of MSTFA and heating for 30 min at 37 °C. Samples were kept at 25 °C for 2 h before injection into the GC/MS. 1 μl of the sample was injected into GC/MS with a split ratio of 1:25. The lower portion, the non-polar chloroform-phase, was transferred to a new vial and 0.90 ml of chloroform and 1 ml of methanol containing 3% v/v H_2SO_4 were added. Lipids and free fatty acids found in samples were transmethylated for 4 h at 100 °C. Each sample was cleaned twice by adding 4 ml of pure water, vortexed and centrifuged at 3800 rpm for 15 minutes. The water phase was discarded and anhydrous sodium sulphate was added to the remaining non-polar extract to remove excess of water. The supernatant was transferred to a new

glass tube and dried by using Speed Vac. 80 µL of chloroform was added to dissolve the dried metabolites. The samples were derivatized and silylated by adding 10 µL of MSTFA and 10 µL of pyridine at 37 °C for 30 min. 1 µL of the sample was injected into the GC/MS with a split ratio of 1:5. Before injection in to the GC/MS, the Lee's retention time index standards (chrysene, naphthalene, and phenanthrene) were added to both polar and nonpolar samples (Eckel, 2000).

GC/MS analysis: Samples were transferred to an auto sampler that injected 1 µl into the GC injection port (model 2100 T, Varian®, Canada) connected to a GC/MS (GC 3400XC with Voyager® ion trap mass analyzer; Varian ®, QC, Canada). The GC was equipped with a capillary column (30 m DB-5MS column with 0.25 mm diameter, 0.25 µm film thickness, Supelco, Canada). The injection port was heated to 230 °C. Helium was used as a carrier gas with a flow rate of 1 ml s^{-1}. The oven was programmed at 70 °C for 5 min and then increased at a rate of 5 °C min^{-1} until 290 °C, where it was held for 6 min. The mass spectra from 45 to 600 *m/z* were recorded using ion trap analyzer. Data was analyzed using Saturn Lab Software. The GC/MS data consisted of scans and abundances of mass ions.

4.3.6. Data processing

The GC/MS output on abundances of mass ions at different scans were imported into spreadsheet and organized using the Pivot Table Procedures of the EXCEL® program. The consistency of mass ion spectra of peaks, with about the same retention time, in five replicates of each treatment was inspected using SATURN Lab software (SATURN ® GC/MS workstation version 5.52), and the most probable choice of a name

was selected for the compound using NIST (National Institute of Standards and Technology, MD, USA) and/or SATURN libraries. Some peaks even though consistent had low probability match. These were designated as unidentified and their spectra (*m/z*) in decreasing order of relative abundance were given in place of names. Metabolites that were not consistent among replicates were excluded from further analysis.

The Lee Retention Index (RI) was calculated for all the metabolites (Lee et al., 1979; Eckel, 2000) using the formula: $RI = \{100 \times (RT_{Unknown} - RT_n)/ (RT_{n+1} - RT_n)\} + 100$ (n), where $RT_{unknown}$ is the Retention Time of the unknown metabolite; the RT_n and RT_{n+1} are the retention time of the standards that eluted before and after the unknown, respectively; (n) represents the number of rings of the aromatic hydrocarbon standards, that is 2 for naphthalene, 3 for phenanthrene and 4 for chrysene. The Lee RI was also used to confirm or to refuse the compound names, proposed by automated mass spectral search libraries, based on the boiling point in degrees Celsius of a metabolite, which is always greater than its RI for metabolites smaller than Hexacosane (Eckel, 2000). Metabolites with boiling points (obtained from SciFinder ® Scholar Version 2002, American Chemical Society) less than their RI were considered unidentified. The tentatively identified metabolites were grouped according to their chemical functions. The metabolite spectra were further compared with Golm Metabolome Database (http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/gmd.html) (Kopka et al., 2005).

4.3.7. Experimental design and data analysis

Three experiments were conducted simultaneously: *1) Metabolite profiling:* the experiment was designed as a randomized complete block with four treatments: water

inoculated and incubated for 24 h (W1=Control), and pathogen inoculated and incubated for 24 h (P1), 48 h (P2) and 96h (P4). The entire block was repeated 5 times, over time, thus 5 replicates. Each experimental unit consisted of 12 leaflets discs cut from six inoculated leaflets of a single-tuber-plant. Each experimental unit consisted of a pool of metabolites from polar (methanol-water) and non-polar (chloroform) extracts analyzed separately using GC/MS. *2) Disease severity assessment:* the experiment was designed as a randomized complete block design with one treatment of pathogen inoculation and conducted five times, thus 5 blocks. Each experimental unit consisted of measurements (over time) of 12 inoculated sites in six leaflets from a single-tuber-plant. *3) Sporulation assessment:* the experiment was a randomized complete block design with one treatment and 5 replicates and 4 blocks over time. Each experimental unit consisted of 10 inoculation sites in five leaflets from a single-tuber-plant.

The data on abundances of metabolites were subjected to analysis of variance using ANOVA procedure of SAS to identify the compounds significantly different among treatments and Duncan's multiple range test at (P=0.05) was used to compare the different treatments (Khattree and Naik, 2000). The metabolites that were significantly increased or decreased in abundances following pathogen inoculation, relative to water inoculation, were identified. The abundances of 106 metabolites were further subjected to FACTOR procedure of SAS, using principle component and the orthogonal (VARIMAX) rotational methods, to identify the contribution (factor-loading) of each metabolite to a treatment and the relationships among treatments based on a spatial location of treatments in scatter plot of factor-scores for significant factors (Johnson and Wickern, 2002; Hamzehzarghani et al., 2005). A positive factor-score was associated

with positive factor-loadings of a set of metabolites. The factor-loading of a compound increased with an increase in its abundance relative to other metabolites. The data on disease severity (average lesion area in mm^2) and sporulation (number of spores $lesion^{-1}$) were subjected to ANOVA using SAS program.

4.4. Results

4.4.1. Disease severity and amount of sporulation

The average lesion areas on 2, 4, 6 and 8 day after inoculation (DAI) were 77.8, 114.3, and 124.5 and 224.3 mm^2, respectively. The different treatments were found to be significantly different at $P \leq .05$ according to the ANOVA results of SAS. The lesion mean areas at day 4 and 6 were significantly different from the other two treatments (Appendix 3. A).The average numbers of sporangia at 6 DAI were not found to be significantly different at 0.05 levels for both the replicates and the blocks. The average number of sporangia was found to be 5.33×10^3 $disc^{-1}$ (Appendix 3.B).

4.4.2. Temporal dynamics of metabolites

4.4.2.1. Homeostasis and PR-metabolites

Metabolite profiling detected more than 300 peaks, however, only a total of 106 peaks were consistent in all the 5 replicates and had abundances $>2x10^3$, including 36 from the chloroform phase and 70 from the methanol-water phase. Among the consistent metabolites 95 were tentatively identified and the remaining 11 were un-identified (Table 4.1). Out of 106 metabolites the abundances of 42 were significantly different (P=0.05) among treatments (W1, P1, P2 and P4). The abundances of most of the

metabolites increased following pathogen inoculation, meaning the ratio of abundances of compounds between Pathogen/Water (P/W-ratio) inoculated were greater than 1.0. The metabolites significantly increased or decreased after the pathogen inoculation were designated as pathogenesis related (PR) metabolites, either increased (PRI) or decreased (PRD) in abundances, respectively (Table 4.1).

4.4.2.2. The temporal dynamics of PR-metabolites within functional groups

The PR-metabolites consisted of 9 amino acids (AAs), 9 fatty acids (FAs), 10 organic acids (OAs), 4 sugars (SRs), 3 to other groups (OG) and 7 Unidentified. Several AAs and OAs were increased in abundances in the early stages of pathogen infection (P1 and P2). The abundances of most of the AAs of aspartate, serine, and glutamate and alanine families were highly increased in P1 treatment, slightly reduced in P2 and highly reduced in P4 treatment (P/W-ratios were P1/W > P2/W > P4/W). On the contrary, the abundances of the aromatic AAs (tyrosine and phenylalanine) and L-Glutamic acid (glutamate family) that were slightly increased in P1 treatment were highly increased in P2 and the P/W-ratios were: P2/W > P1/W > P4/W. Two unidentified metabolites (38 and 40; serial numbers in Table 4.1) and D-Glucose (20) were significantly decreased in abundances following pathogen inoculation (1, 2 and 4 DAI).

Among sugars, only D-Glucose (20) was a PR-metabolite in P1. In P2, two PRD-metabolites and one PRI-metabolite were detected, namely D-Glucose (20), Myo-Inositol (33) and Xylulose (42), respectively. In P4, 3 PRD-metabolites and 1 PRI-metabolite were detected, namely D-Glucose (20), Myo-Inositol (33), D-Mannitol (21) and Xylulose (42), respectively.

Ten organic acids were found to be PR-metabolites. In P1, 4 PRI-metabolites including propanetricarboxylic acid (15=Isocitric acid), Butanoic acid (18), trihydroxypentanoic acid (24), and galactonic acid (25) were detected. In P2, 6 PRI-metabolites were detected including trihydroxybutyric acid (16), 2-Keto-l-gluconic acid (17), D-Gluconic acid (19), tetronic acid (23), trihydroxypentanoic acid (24), and galactonic acid (25). In P4, 6 OAs were PRI-metabolites including dihydroxybutanoic acid (14), 2-Keto-l-gluconic acid (17), D-Gluconic acid (19), tetronic acid (23), galactonic acid (25) and L-Gluconic acid (28). Of these OAs only dihydroxybutanoic acid (14) was a PRD-metabolite.

Three fatty acids were increased in abundances in P1, namely 9,12-Octadecadienoic acid (5= Linoleic acid), 9-Octadecanoic acid (7= Oleic acid) and Hexadecanoic acid (10= Palmitic acid) with P/W-ratio of P1/W > P2/W > P4/W. In P2, 3 PRI-metabolites and 2 PRD-metabolites were detected, namely 9-Octadecenoic acid (7), Eicosanoic acid (8), Hexadecanoic (9), 7,10,13-Hexadecatrienoic acid (2), 7,10-Hexadecadienoic acid (3), respectively. In P4, 4 PRD-metabolites and 1 PRI-metabolite, namely 7,10,13-Hexadecatrienoic acid (2), 7,10-Hexadecadienoic acid (3), 9,12,15-Octadecatrienoic acid (4), 9-Hexadecenoic acid (6), and Eicosanoic acid (8), were found. The total abundances of the unsaturated FAs were reduced with the progress of the disease and the P/W-ratios were: P4/W < P2/W < P1/W.

4.4.3. Factor-loadings of metabolites to factor vectors and their plausible plant defense phases

The first three factors explained 100% of the total variance (F1=45.56%, F2=27.47% and F3=26.97%) among four treatments based on factor analysis of abundances of 106 consistent metabolites. The scatter plot, using factor-scores for the first three factors, discriminating four treatments (W1, P1, P2 and P4) is shown in Fig. 4.1. Factor-loadings of different compounds to each of the three factors are shown as a list in Table 4.1 and as part of the metabolic pathway in Fig. 4.2. The relative factor-scores of the first three factors discriminating different treatments, and the sets of metabolites with significant factor-loadings to each factor vector were used to identify four plausible plant-pathogen interaction functions following pathogen inoculation:

4.4.3.1. Factor 1. Homeostasis (no pathogen stress = W1)

The treatments in a descending order of F1-scores were: W1> P1> P2> P4. The treatment W1 = at 1 DAI, had the highest positive F1 score, and accordingly the compounds with significant loading to F1-vector were used to explain the normal plant function or the homeostasis. Thirty-three metabolites had significant factor-loadings to F1 vector (Factor-loadings > +0.50; Table 4.1), with 11 SRs (in descending order of factor-loading = 89, 33, 95, 87, 20, 90, 21, 84, 74, 75, and 66), 8 OAs (68, 71, 98, 14, 70, 88, 81, and 96), 4 FAs (3, 2, 4, 6), 2 AAs (22, 101), 5 unidentified (UI) (38, 103, 12, 40, and 105) and 3 other groups (OG) (1, 99, and 43). Of these metabolites 13 were PR-metabolites.

4.4.3.2. Factor 2. Primary defense response or initial infection = P1

The treatments in a descending order of F2-scores were: P1> P4> P2> W1. The treatment P1 = at 1 DAI, had the highest F2-score and accordingly the metabolites with significant loadings to F2, which included several plant defense related metabolites, were considered to explain the primary defense response. Thirty-four metabolites loaded significantly to F2, including 9 AAs (27, 34, 94, 92, 22, 30, 32, 29, and 93), 7 FAs (51, 65, 10, 5, 7, 58, and 6), 7 OAs (18, 15, 24, 81, 69, 80, and 79), 2 SRs (85, 73), 6 OG (102, 1, 45, 59, 11, and 43), and 3 UI (39, 37, and 12). Also, of these 34 metabolites, 18 were PR-metabolites consisting of 6 AAs (27, 34, 22, 30, 32, and 29), 3 FAs (10, 5, and 7), 3 OAs (18, 15, and 24,), 2 UI (39 and 37), and 1 OG (11).

4.4.3.3. Factor 3. Secondary defense response or advanced infection = P2

The treatments in a descending order of F3-scores were: P2> P1> W1> P4. The treatment P2 = at 2 DAI, had the highest F3-score, and accordingly the metabolites with significant loadings to F3, which included several plant defense related metabolites, were used to explain the secondary defense response. Thirty-three metabolites loaded significantly to F3, including 7 FAs (57, 9, 54, 63, 58, 52, and 7), 6 AAs (31, 35, 32, 26, 93, and 29), 6 OAs (67, 16, 80, 79, 25, and 15), 4 SRs (78, 77, 66, and 73), 5 UI (41, 104, 106, 37, and 13) and 5 OG (55, 36, 102, 11, and 99). Of these metabolites, 15 were PR-metabolites including 5 AAs (31, 35, 32, 26, and 29), 2 OAs (16 and 25), 2 FAs (9, and 7), 3 UI (41, 37, and 13), and 2 OG (36, and 11).

4.4.3.4. Factors 1-3. Collapse of the primary and secondary defense responses or disease establishment = P4

The treatment P4 = at 4 DAI, had the lowest scores for all the three factors and accordingly the metabolites with significantly negative factor-loadings, included negative factor-loading of defense related metabolites, were used to explain the function of collapse of defense responses. Only 6 metabolites including 2 OAs (97, and 28), 2 FAs (62, and 64), and 2 SRs (83, and 86) had negative loading to F1, F2, F3 and their P/W ratios were: P4/W >P2/W > P1/W. Among the metabolites significantly loaded only L-Gluconic acid (28) was a PR-metabolite.

Table 4.1. Metabolites and their abundances ($x10^6$) detected in leaves of potato cultivar AC Novachip at different incubation times after inoculation with the pathogen *P. infestans* or water (control).

No.	Ex[1]	RI[2]	Metabolite Name[3]	CAS[4]	Grp[5]	W[6]	P1/W[7]	P2/W	P4/W	F1[8]	F2	F3
1	C	310.0	3,7,11,15-Tetramethyl-2-hexadecen (N)	102608-53-7	O(AA)	0.17^{AB}	1.13^{A}	0.73^{B}	0.67^{B}	0.83	0.55	-0.06
2	C	318.3	7,10,13-Hexadecatrienoic acid, me (N)	56554-30-4	FA	2.04^{A}	1.02^{A}	0.76^{B}	0.47^{C}	0.92	0.38	0.10
3	C	317.5	7,10-Hexadecadienoic acid, meth (N)	16106-03-9	FA	0.25^{A}	1.04^{A}	0.71^{B}	0.45^{C}	0.93	0.36	0.08
4	C	350.0	9,12,15-Octadecatrienoic acid, (GN)	301-00-8	FA	16.96^{AB}	1.06^{A}	0.87^{B}	0.66^{C}	0.89	0.39	0.23
5	C	349.0	9,12-Octadecadienoic acid (Z,Z) (NG)	112-63-0	FA	8.33^{BC}	1.23^{A}	1.06^{B}	0.92^{C}	0.41	0.81	0.42
6	C	322.3	9-Hexadecenoic acid, methyl ester, (N)	1120-25-8	FA	0.60^{A}	1.07^{A}	0.96^{B}	0.80^{B}	0.80	0.50	0.33
7	C	350.8	9-Octadecenoic acid (Z)-, methyl (N)	112-62-9	FA	0.11^{C}	1.50^{A}	1.45^{AB}	1.19^{BC}	-0.26	0.75	0.61
8	C	382.1	Eicosanoic acid, methyl ester (NG)	1120-28-1	FA	0.90^{B}	1.10^{AB}	1.23^{A}	1.24^{A}	-0.92	-0.01	0.40
9	C	328.7	Hexadecanoic acid (N)	57-10-3	FA	0.23^{B}	1.50^{B}	2.46^{A}	1.06^{B}	-0.15	0.02	0.99
10	C	323.3	Hexadecanoic acid, methyl ester (N)	112-39-0	FA	7.12^{B}	1.19^{A}	1.08^{AB}	1.03^{B}	0.05	0.93	0.37
11	C	473.1	Solanid-5-en-3-ol, (3á)- (N)	80-78-4	O(G)	0.42^{B}	2.17^{A}	2.13^{A}	1.70^{A}	-0.45	0.63	0.64
12	C	313.6	Unidentified (123,81,95,278,96) (NG)	N/A	N/A	0.38^{A}	1.06^{A}	0.72^{B}	0.65^{B}	0.84	0.51	-0.20
13	C	458.7	Unidentified (193,73,119,105,147) (NG)	N/A	N/A	1.30^{B}	1.39^{AB}	1.99^{A}	1.83^{A}	-0.85	-0.06	0.52
14	M	243.3	(R*,S*)-3,4-Dihydroxybutanoic acid (N)	55191-53-2	OA	0.08^{A}	0.76^{AB}	0.74^{AB}	0.59^{B}	0.95	-0.30	-0.06

15	M	293.3	1,2,3-Propanetricarboxylic acid, 2- (N)	14330-97-3	OA	0.77^{B}	1.50^{A}	1.31^{AB}	1.01^{B}	0.13	0.84	0.52
16	M	264.8	2,3,4-Trihydroxybutyric acid tetra- (N)	38191-88-7	OA	0.88^{B}	1.34^{AB}	1.52^{A}	1.14^{B}	-0.25	0.43	0.87
17	M	296.2	2-Keto-l-gluconic acid, penta(trim (GN)	N/A	OA	0.21^{B}	1.28^{B}	1.60^{A}	1.69^{A}	-0.95	0.05	0.29
18	M	259.1	Butanoic acid, 4-[bis(trimethylsilyl) (NG)	39508-23-1	OA	4.49^{B}	1.53^{A}	1.23^{B}	1.12^{B}	-0.01	0.94	0.33
19	M	330.3	D-Gluconic acid, 2,3,4,5,6-pentakis (N)	34290-52-3	OA	0.08^{B}	1.12^{B}	1.48^{A}	1.69^{A}	-0.98	-0.20	0.08
20	M	362.7	D-Glucose, 4-O-[2,3,4,6-tetrakis-O (N)	55669-93-7	SR	0.20^{A}	0.69^{B}	0.67^{B}	0.54^{B}	0.93	-0.31	-0.21
21	M	322.9	D-Mannitol, 1,2,3,4,5,6-hexakis-O- (GN)	14317-07-8	SR	0.14^{AB}	1.12^{A}	0.96^{B}	0.70^{C}	0.86	0.40	0.32
22	M	229.6	EIQTMS_N12C_L....L-Alanine (3TMS) (G)	N/A	AA	0.71^{B}	1.77^{A}	0.81^{B}	0.38^{B}	0.61	0.78	0.15
23	M	261.8	EIQTMS_N12....[Tetronic acid (4TMS)] (G)	N/A	OA	0.13^{C}	1.17^{C}	1.72^{B}	2.11^{A}	-0.98	-0.17	0.08
24	M	278.9	EIQTMS..2,4,5-Trihydroxypentanoic acid (G)	N/A	OA	0.44^{B}	1.37^{A}	1.26^{A}	1.18^{AB}	-0.35	0.82	0.45
25	M	333.5	Galactonic acid, 2,3,4,5,6-pentaki (GN)	55400-16-3	OA	0.10^{C}	1.64^{B}	1.96^{A}	1.60^{B}	-0.68	0.32	0.66
26	M	274.6	Glutamine, tris(trimethylsilyl)- (GN)	15985-07-6	AA	0.32^{B}	2.47^{A}	2.96^{A}	2.33^{A}	-0.63	0.38	0.68
27	M	258.1	L-Aspartic acid, N-(trimethylsilyl)-, (GN)	55268-53-6	AA	1.24^{B}	2.50^{A}	1.67^{B}	1.03^{B}	0.12	0.88	0.46
28	M	299.1	L-Gluconic acid, 2,3,5,6-tetrakis-O (N)	56298-43-2	OA	0.23^{BC}	0.85^{C}	1.07^{B}	1.32^{A}	-0.81	-0.54	-0.24
29	M	219.4	L-Isoleucine, N-(trimethylsilyl)-, tri (NG)	7483-92-3	AA	0.24^{B}	3.43^{A}	3.29^{A}	2.60^{B}	-0.52	0.63	0.57
30	M	257.6	L-Proline, 5-oxo-1-(trimethylsilyl)-, tri (NG)	30274-77-2	AA	1.77^{BC}	1.60^{A}	1.22^{AB}	0.71^{C}	0.48	0.76	0.45
31	M	324.5	L-Tyrosine, N,O-bis(trimethylsilyl)-, tri (NG)	51220-73-6	AA	0.24^{B}	2.36^{A}	2.62^{A}	1.31^{B}	-0.12	0.49	0.86
32	M	203.9	L-Valine, N-(trimethylsilyl)-, trimeth (NG)	7364-44-5	AA	0.54^{B}	2.68^{A}	2.21^{A}	1.19^{B}	0.01	0.73	0.68

33	M	347.6	Myo-Inositol, 1,2,3,4,5,6-hexakis-O (GN)	2582-79-8	SR	0.81^{A}	0.95^{AB}	0.77^{BC}	0.66^{C}	0.97	0.23	-0.09
34	M	234.7	N,O,O-Tris(trimethylsilyl)-L-threonine (GN)	7537-02-2	AA	0.36^{C}	3.04^{A}	2.09^{B}	1.27^{BC}	0.04	0.88	0.48
35	M	274.8	N,O-Bis(trimethylsilyl)-L-phenylalanine (GN)	7364-51-4	AA	0.42^{B}	1.51^{AB}	1.82^{A}	1.43^{AB}	-0.56	0.26	0.79
36	M	337.9	Silanamine, 1,1,1-trimethyl-N-(trim (GN)	55556-99-5	O(P)	0.09^{C}	1.44^{B}	1.97^{A}	1.08^{C}	-0.15	0.07	0.99
37	M	242.0	Unidentified (174,73,175,155,86) (NG)	N/A	N/A	0.56^{B}	1.38^{A}	1.32^{A}	0.90^{B}	0.26	0.59	0.77
38	M	240.5	Unidentified (189,73,221,147,174) (NG)	N/A	N/A	0.21^{A}	0.80^{B}	0.75^{BC}	0.64^{C}	0.96	-0.23	-0.15
39	M	299.6	Unidentified (73,288,147,74,361) (NG)	N/A	N/A	3.63^{B}	1.48^{A}	1.16^{B}	0.95^{B}	0.27	0.88	0.38
40	M	253.0	Unidentified (73,333,147,265,407) (NG)	N/A	N/A	42.96^{A}	0.64^{B}	0.66^{B}	0.65^{B}	0.72	-0.57	-0.39
41	M	167.9	Unidentified (73,89,147,59,161) (NG)	N/A	N/A	5.62^{BC}	1.15^{AB}	1.19^{A}	0.94^{C}	0.20	0.42	0.88
42	M	268.0	Xylulose tetra-TMS (GN)	N/A	SR	0.36^{C}	1.29^{BC}	1.70^{AB}	2.14^{A}	-0.99	-0.10	0.04
43	C	260.9	2,4,6-Tri-t-butylbenzenethiol (GN)	961-39-7	O(SC)	0.33	1.05	0.95	0.94	0.74	0.62	-0.25
44	C	248.8	2,5-Cyclohexadiene-1,4-dione, 2,6 (N)	719-22-2	O(DQ)	0.18	1.12	1.17	1.23	-1.00	0.08	0.02
45**	C	311.5	3,5-Cyclohexadiene-1,2-dione, 3,5 (N)	3383-21-9	O(DQ)	0.44	1.38	1.11	1.33	-0.57	0.80	-0.21
46	C	493.1	á-Sitosterol acetate (N)	915-05-9	O(S)	2.93	1.18	1.24	1.38	-0.97	0.23	0.06
47	C	506.3	á-Sitosterol trimethylsilyl ether (NG)	2625-46-9	O(S)	1.34	1.00	1.18	1.24	-0.92	-0.36	0.15
48	C	210.8	Benzene, 1,3-bis(1,1-dimethylethyl (NG)	1014-60-4	O(AH)	0.21	1.16	1.20	1.22	-0.86	0.29	0.41
49	C	467.4	Cholest-5-ene, 3-methoxy-, (3á)- (N)	1174-92-1	O(S)	0.34	0.67	1.23	1.10	-0.39	-0.87	0.29

50**	C	408.0	Docosanoic acid, methyl ester (N)	929-77-1	FA	0.62	0.98	1.14	1.21	-0.89	-0.46	0.09
51	C	338.8	Heptadecanoic acid, methyl ester (NG)	1731-92-6	FA	0.26	1.11	0.98	0.98	0.23	0.97	0.01
52**	C	334.4	Hexadecanoic acid, 14-methyl-, m (N)	2490-49-5	FA	0.25	1.02	1.12	1.09	-0.74	-0.09	0.66
53	C	415.0	Hexadecanoic acid, 2,3-bis[(trime (N)	1188-74-5	FA	0.27	4.76	5.27	5.64	-0.84	0.42	0.34
54	C	341.7	Hexadecanoic acid, trimethylsilyl (NG)	55520-89-3	FA	0.88	1.22	1.45	0.94	0.05	0.21	0.98
55	C	287.1	Nonadecane (N)	629-92-5	O(AK)	0.15	1.08	1.20	0.98	-0.09	0.01	1.00
56	C	437.7	Octadecanoic acid, 2,3-bis[(trime (N)	1188-75-6	FA	1.42	3.13	3.48	4.03	-0.91	0.33	0.25
57	C	354.0	Octadecanoic acid, methyl ester (GN)	112-61-8	FA	7.73	1.01	1.05	0.98	0.09	-0.03	1.00
58	C	370.4	Octadecanoic acid, trimethylsilyl (NG)	18748-91-9	FA	1.27	1.17	1.20	0.99	0.06	0.57	0.82
59	C	319.5	Propionic aldehyde, 2',3,3,4',5' (N)	N/A	O(Q)	0.62	1.44	1.15	1.40	-0.61	0.77	-0.20
60	C	471.1	Stigmastan-3,5-dien (N)	N/A	O(S)	0.42	1.48	1.47	1.81	-0.95	0.30	0.01
61	C	496.1	Stigmasterol trimethylsilyl ether (NG)	14030-29-6	O(S)	1.09	1.11	1.11	1.19	-0.97	0.23	-0.09
62**	C	432.3	Tetracosanoic acid, methyl ester (N)	2442-49-1	FA	0.52	0.98	0.99	1.10	-0.73	-0.24	-0.64
63**	C	510.2	Triacontanoic acid, methyl ester (N)	629-83-4	FA	0.63	0.98	1.30	0.88	0.14	-0.25	0.96
64**	C	420.4	Tricosanoic acid, methyl ester (N)	2433-97-8	FA	0.13	0.89	0.99	1.21	-0.71	-0.57	-0.40
65**	C	291.0	Tridecanoic acid, 12-methyl-, meth (N)	5129-58-8	FA	0.18	1.30	1.12	1.14	-0.20	0.96	0.20
66	M	297.3	.beta.-DL-Arabinopyranose, 2 (N)	56271-64-8	SR	0.50	1.03	1.09	0.86	0.57	0.07	0.82
67	M	304.5	1-Cyclohexene-1-carboxylic a (N)	55520-78-0	OA	0.36	1.29	1.57	0.99	-0.01	0.18	0.98

68	M	305.7	1-Propene-1,2,3-tricarboxylic acid, (N)	55530-71-7	OA	5.28	0.83	0.74	0.55	1.00	-0.05	0.00
69	M	228.9	2-Butenedioic acid (E)-, bis(trime (GN)	17962-03-7	OA	0.35	1.12	0.87	0.99	0.24	0.74	-0.63
70	M	249.4	3-Hydroxy glutaric acid tri-TMS (N)	N/A	OA	0.34	0.81	0.79	0.71	0.91	-0.38	-0.13
71	M	263.6	á-Amino isobutyric acid tri-TMS II (N)	N/A	OA	0.07	0.97	0.92	0.81	0.97	0.04	0.22
72	M	179.6	Acetic acid, [(trimethylsilyl)oxy]-, (N)	33581-77-0	OA	1.33	1.31	1.31	1.32	-0.87	0.44	0.23
73	M	283.4	á-D-Galactofuranoside, ethyl 2,3,5, (N)	55493-81-7	SR	0.71	1.28	1.22	1.02	0.02	0.71	0.70
74	M	255.0	à-D-Galactopyranoside, methyl 2 (N)	56196-94-2	SR	0.56	0.62	0.77	0.62	0.73	-0.68	-0.04
75	M	235.4	á-D-Galactopyranoside, methyl 2,3- (N)	56211-10-0	SR	0.11	0.66	0.73	0.71	0.69	-0.66	-0.30
76	M	420.9	à-D-Glucopyranoside, 1,3,4,6-tetra (N)	19159-25-2	SR	0.52	1.24	1.55	1.66	-0.96	-0.07	0.27
77	M	285.3	à-L-Mannopyranose, 6-deoxy-1,2,3, (N)	55057-21-1	SR	0.09	1.23	1.48	1.11	-0.29	0.18	0.94
78	M	280.0	Arabinofuranose, 1,2,3,5-tetrakis-O (N)	55399-49-0	SR	0.29	1.30	1.42	0.97	0.06	0.33	0.94
79	M	291.9	Arabinoic acid, 2,3,5-tris-O-(trimeth (N)	10589-37-4	OA	0.20	1.17	1.14	0.90	0.45	0.58	0.68
80	M	168.9	Butanedioic acid, 2,3-bis[(trimethyls (N)	57456-93-6	OA	2.74	1.32	1.32	1.01	-0.02	0.59	0.81
81	M	222.4	Butanedioic acid, bis(trimethylsilyl) (GN)	40309-57-7	OA	1.39	1.11	0.91	0.95	0.64	0.74	-0.20
82	M	190.6	Cystathionine-diTMS (N)	73090-79-6	O(AD)	1.00	0.94	0.75	0.98	0.22	0.06	-0.97
83	M	339.1	D-Altrose, 2,3,4,5,6-pentakis-O-(trim (N)	56114-56-8	SR	0.10	1.07	1.03	1.14	-0.90	-0.03	-0.43
84	M	315.5	D-Fructose, 1,3,4,5,6-pentakis-O-(tri (N)	56196-14-6	SR	11.44	1.03	0.87	0.66	0.85	0.46	0.26
85	M	281.1	D-Ribofuranose, 1,2,3,5-tetrakis-O-(tr (N)	56271-69-3	SR	0.14	1.38	1.17	1.04	0.00	0.95	0.32

86	M	292.7	D-Xylofuranose, 1,2,3,5-tetrakis-O-(tr (N)	56271-68-2	SR	0.59	0.84	0.98	0.98	-0.13	-0.98	-0.15
87	M	289.2	EIQTMS_N12C_SD1_1727.8_1215BK (G)	N/A	SR	0.13	0.81	0.81	0.74	0.94	-0.33	-0.05
88	M	231.9	Erythro-Pentonic acid, 2-deoxy-3,4, (N)	74742-30-6	OA	0.17	0.67	0.75	0.54	0.91	-0.41	0.05
89	M	320.8	Galactose oxime hexaTMS (NG)	120850-88-6	SR	2.29	0.97	0.83	0.69	0.98	0.20	0.02
90	M	317.9	Glucose oxime hexaTMS (N)	120850-89-7	SR	12.87	0.98	0.81	0.72	0.91	0.37	-0.18
91	M	221.0	Glycine, N,N-bis(trimethylsilyl)-, tri (GN)	5630-82-0	AA	0.58	1.12	1.01	1.37	-0.79	0.20	-0.58
92	M	184.0	l-Alanine, N-(trimethylsilyl (NG)	27844-07-1	AA	1.28	1.98	1.66	1.45	-0.35	0.83	0.43
93	M	220.1	L-Proline, 1-(trimethylsilyl)-, trimeth (NG)	7364-47-8	AA	0.11	1.89	1.99	1.74	-0.59	0.52	0.62
94	M	230.3	L-Serine, N,O-bis(trimethylsilyl)-, tr (GN)	7364-48-9	AA	0.73	2.88	2.22	2.04	-0.40	0.84	0.36
95	M	260.8	Mannofuranoside, methyl 2,3,5,6- (N)	6737-01-5	SR	0.40	0.85	0.83	0.76	0.95	-0.27	-0.13
96	M	267.6	Pentanedioic acid, 2-(methoxyimi (N)	60022-87-9	OA	0.26	0.74	0.67	0.75	0.62	-0.41	-0.67
97	M	224.6	Propanoic acid, 2,3-bis[(trimethylsi (NG)	38191-87-6	OA	4.23	0.92	1.45	1.96	-0.93	-0.36	-0.12
98	M	176.7	Propanoic acid, 2-[(trimethylsilyl) (NG)	17596-96-2	OA	5.08	0.89	0.89	0.72	0.97	-0.15	0.21
99	M	247.1	Putrescine tetraTMS (GN)	39772-63-9	O(NC)	1.18	0.99	0.99	0.88	0.82	0.14	0.55
100	M	294.5	Ribonic acid, 2,3,4,5-tetrakis-O-(tr (NG)	57197-35-0	OA	0.13	0.93	1.19	1.16	-0.74	-0.62	0.25
101	M	282.3	TMS 1-TMS-5-TMSoxy-3-(2-TMSa- (N)	69937-47-9	AA	1.09	0.61	0.63	0.71	0.58	-0.60	-0.55
102	M	271.2	Trimethylsilyl ether of glycerol (NG)	6787-10-6	O(A)	0.15	1.51	1.52	0.96	0.15	0.55	0.82
103	M	313.9	Unidentified (218,73,217,74,147) (NG)	N/A	N/A	15.71	1.05	0.89	0.67	0.93	0.26	0.25

104	M	262.6	Unidentified (247,249,173,175,145) (NG)	N/A	N/A	0.20	1.25	1.30	1.09	-0.15	0.46	0.87
105	M	284.2	Unidentified (290,73,217,234,304) (NG)	N/A	N/A	0.25	0.80	0.86	0.85	0.63	-0.69	-0.36
106	M	311.6	Unidentified (346,347,73,74,345) (NG)	N/A	N/A	2.80	1.12	1.14	0.85	0.46	0.31	0.83
Percentage of Variance explained										45.56	27.47	26.97

1 Ex = Extract; methanol-water (M) and chloroform (C).

2 Retention Index (RI), calculated based on Eckel (2000); ** Metabolites having Boiling Points < Lee Retention Index but had high match probabilities in the libraries

3 Shortened names according to NIST (N = a letter in parenthesis at the end of the name) or GOLM Metabolome Database (G) or both (GK), libraries with higher probability for the same metabolite is listed first.

4 CAS Registry Number = Chemical Abstract Service Registry Number.

5 Chemical groups of compounds: AA= Amino Acid; FA= Fatty Acid; OA= Organic Acid; OAA= Other Aliphatic Alcohol; O (AD)= Other amino acid derivatives; O (AH)= Other Aromatic Hydrocarbon; O (D)= Other Diene; O (P)= Other Phenolic; O (DQ)= Other Diene; O (G)= Other Glykoalkaloids; O (Q)= Other Quinine; O (S)= Other Steroid; O (SC)= Other Sulfur containing; ST= Steroids; Sugar=SR.

6 Total abundance ($x10^6$) of Water inoculated plants (control).

7 The metabolite regulation ratio = pathogen inoculated over water inoculated (P/W>1.0 is increased in abundances) at 1, 2 and 4 DAI; different letters indicate significance at P=0.05 by comparing the means of the total abundance of the four treatments using Duncan's multiple range test.

8 Factor-loadings of metabolites to F1, F2 and F3-scores, based on factor analysis of abundances of 106 significant metabolites; the loadings can be positive or negative.

Fig. 4.1. Scatter plot of treatments using factor scores based on factor analysis of the abundances of 106 metabolites. The treatments are: Water inoculated 1 DAI (W1=♥), Pathogen inoculated 1, 2 and 4 DAI (P1=♣, P2=♦, and P4=♠), respectively. The factors explained the plausible underneath functions (Treatments with the highest F-scores); Treatments with F1-scores in descending order are: W1> P1> P2> P4 = Homeostasis state of plant (no stress); Treatments with F2-scores in descending order are: P1> P4> P2>W1 = Primary defense response; Treatments with F3 scores in descending order are: P2> P1> W1> P4 = Secondary defense response; Treatments with all negative F-scores F1,2,3 =P4 (Collapse of the defense response). The metabolites differentially and significantly loaded to different factors are given.

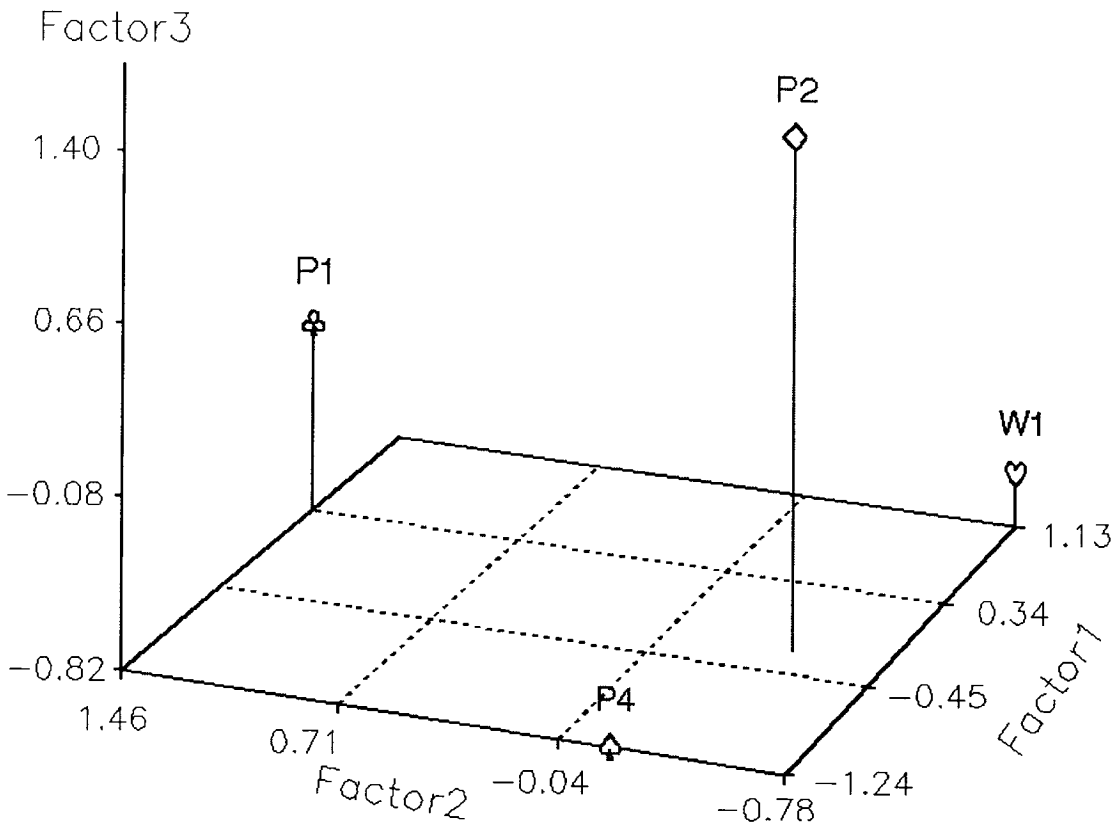
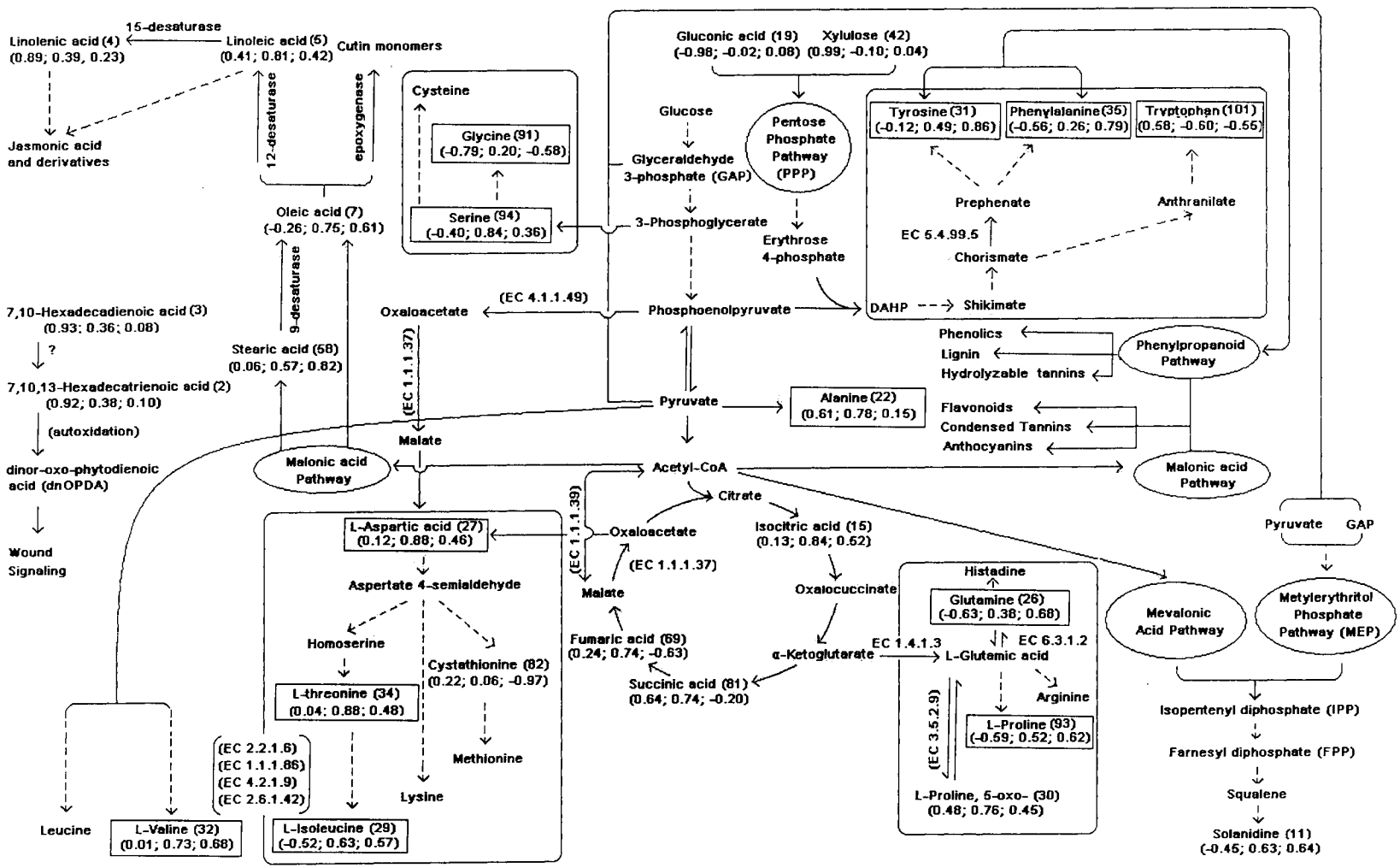

Factor3
1.40
0.66
−0.08
−0.82
P1
P2
W1
P4
1.13
0.34
−0.45
−1.24
Factor1
1.46
0.71
−0.04
−0.78
Factor2

Fig. 4.2. Metabolic pathways of the potato cv. AC Novachip inoculated with *P. infestans*. The Factor loadings of metabolites to F1; F2; F3 vectors are given in the pathway, below the metabolites that are detected in this study, respectively. F1= Homeostasis; F2= Primary defense response; F3= Secondary defense response; All 3 factor vectors low= Collapse of defense. Boxes= detected amino acids; Ellipses= metabolic pathways. EC= Enzyme commission number according to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB). DAHP= 3-deoxy—D-arabino-heptulosonate-7-phosphate. This figure has been adapted mainly from (Coruzzi and Last, 2000 and Taiz and Zeiger 2002).


15-desaturase
Linolenic acid (4)
(0.89; 0.39; 0.23)
Linoleic acid (5)
(0.41; 0.81; 0.42)
Cutin monomers
Jasmonic acid
and derivatives
12-desaturase
epoxygenase
Oleic acid (7)
(-0.26; 0.75; 0.61)
9-desaturase
7,10-Hexadecadienoic acid (3)
(0.93; 0.36; 0.08)
?
7,10,13-Hexadecatrienoic acid (2)
(0.92; 0.38; 0.10)
(autoxidation)
dinor-oxo-phytodienoic
acid (dnOPDA)
Wound
Signaling
Stearic acid (58)
(0.06; 0.57; 0.82)
Cysteine
Glycine (91)
(-0.79; 0.20; -0.58)
Serine (94)
(-0.40; 0.84; 0.36)
Gluconic acid (19)
(-0.98; -0.02; 0.08)
Xylulose (42)
(0.99; -0.10; 0.04)
Glucose
Glyceraldehyde
3-phosphate (GAP)
Pentose
Phosphate
Pathway
(PPP)
3-Phosphoglycerate
Erythrose
4-phosphate
Tyrosine (31)
(-0.12; 0.49; 0.86)
Phenylalanine (35)
(-0.56; 0.26; 0.79)
Tryptophan (101)
(0.58; -0.60; -0.55)
Prephenate
Anthranilate
EC 5.4.99.5
Chorismate
DAHP
Shikimate
(EC 4.1.1.49)
Oxaloacetate
Phosphoenolpyruvate
Phenolics
Lignin
Hydrolyzable tannins
Phenylpropanoid
Pathway
(EC 1.1.1.37)
Pyruvate
Alanine (22)
(0.61; 0.78; 0.15)
Flavonoids
Condensed Tannins
Anthocyanins
Malate
Malonic acid
Pathway
Acetyl-CoA
Malonic acid
Pathway
Citrate
(EC 1.1.1.39)
Oxaloacetate
Isocitric acid (15)
(0.13; 0.84; 0.62)
L-Aspartic acid (27)
(0.12; 0.88; 0.46)
(EC 1.1.1.37)
Malate
Aspartate 4-semialdehyde
Oxalocuccinate
Histadine
Glutamine (26)
(-0.63; 0.38; 0.68)
Homoserine
Fumaric acid (69)
(0.24; 0.74; -0.63)
α-Ketoglutarate
EC 1.4.1.3
EC 6.3.1.2
L-Glutamic acid
Cystathionine (82)
(0.22; 0.06; -0.97)
Succinic acid (81)
(0.64; 0.74; -0.20)
Arginine
L-threonine (34)
(0.04; 0.88; 0.48)
(EC 3.5.2.9)
L-Proline (93)
(-0.59; 0.52; 0.62)
(EC 2.2.1.6)
(EC 1.1.1.86)
(EC 4.2.1.9)
(EC 2.6.1.42)
Methionine
Lysine
L-Proline, 5-oxo- (30)
(0.48; 0.76; 0.45)
Leucine
L-Valine (32)
(0.01; 0.73; 0.68)
L-Isoleucine (29)
(-0.52; 0.63; 0.57)
Pyruvate
GAP
Mevalonic
Acid Pathway
Metylerythritol
Phosphate
Pathway (MEP)
Isopentenyl diphosphate (IPP)
Farnesyl diphosphate (FPP)
Squalene
Solanidine (11)
(-0.45; 0.63; 0.64)

4.5. Discussion

A total of 106 metabolites, including 42 PR-metabolites, were tentatively identified from potato leaves inoculated with late blight pathogen. This is the first report of PR-metabolites in the potato-late blight interaction following metabolomics approach. Factor analysis identified several metabolites associated with different factor-vectors, which in turn discriminated the four treatments (W1, P1, P2 and P4). The factor vectors with the highest factor-scores, along with the respective sets of metabolites with significant factor-loadings and also their previously known role in plant defense against biotic stress, enabled identification of four hidden plant-pathogen interaction functions: i) Homeostasis; ii) Primary defense against pathogen attack; iii) Secondary defense against pathogen attack; iv) Collapse of the host defense. The up or down regulation of the PR-metabolites were further used to explain the role of metabolites in plant defense and also the plausible "scale-free" satellite-metabolic-network of plant following pathogen invasion (Barabasi and Oltvai 2004) using metabolic pathway bioinformatics resources, Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/), and Metabolic Pathways of the Diseased Potato (http://www.scri.sari.ac.uk/TiPP/pps/Chart.pdf). Several metabolites, previously known to be associated with biotic stresses of plants, were significantly increased in abundances in 24 and 48 h following pathogen inoculation, while the abundances of these metabolites decreased in 96 h, meaning collapse of the primary and secondary defense responses. Even though the host-pathogen interaction is quite complex the temporal dynamics, type and abundance, of metabolites detected here following pathogen inoculation could be used to better understand their role in plant pathogenesis and defense.

4.5.1. Homeostasis (F1=W1)

The F1 appears to explain non-stressed or homeostasis state of plant, where plant produces sugars through photosynthesis that are utilized to supply essential energy for the plant growth and the excess sugars are stored for future use. The homeostasis conditions such as photosynthesis, food storage and respiration require the coordination of several metabolic pathways that work under a complete harmony. Some of the amino acids detected at 1 DAI, in addition to their role in the production of small molecular weight defense compounds such as phenols, flavones, coumarines, nitrogen and sulfur containing anti-microbial and insect deterrent compounds such as glucosinolates and glycosides (Osbourn, 1996a), they are also important in the production of structural proteins such as microfilaments or actin filaments that are reported to be involved in the defense response against *P. infestans* attack (Furuse et al., 1999). Furthermore, they are important in the production of microtubules, enzymes and PR-proteins that play important roles in plant-pathogen interactions (van Loon and Van Strien, 1999).

Fatty acids are major components of the triglycerides, cutin, suberin, and waxes, plasma, plastid and mitochondrial membranes (Somerville et al, 2000). The unsaturated FAs are the basic components of the membrane lipids and make about 70% of the membrane lipids of the chloroplasts (Yaeno et al., 2004). In Water treatment, all of the 4 FAs that loaded highly to F1 (homeostasis) were unsaturated. However, following pathogen invasion, over time, their total abundances were reduced. In these cases, either the production of the unsaturated FAs was reduced, as a result of the malfunctioning of the chloroplasts due to pathogen infection (Soulie et al., 1989) or the utilization of the unsaturated FAs was increased.

4.5.2. Primary defense (F2=P1)

Following pathogen attack, only a few sugars loaded significantly either to the primary or to the secondary defense responses of the plant. The abundances of D-Glucose (20) and two unidentified metabolites (38 and 40; appear to be sugars) were decreased in treatments following pathogen inoculation. Broeckling et al. (2005) found that the carbon resources of the plant especially sucrose were highly reduced after the exposure of cell culture of *Medicago truncatula* to different elicitors including yeast, methyl jasmonate and UV light. Sugars are precursors of many metabolic pathways and are the building blocks of cell walls, middle lamella, and also they participate in the post modification of proteins and fatty acids. Also, they are important in the production of structural defense materials such as callose and papillae in response to pathogens attack. The deposition of callose ((1-3) β-D-glucan chains) following infection by *P. infestans* in clones of *Solanum* has been reported (Vleeshouwers et al. 2000). The callose accumulation is mainly associated with the deposition of papillae and was reported in biotrophic and hemibiotrophic fungi and it preceded the hypersensitive response (Schmelzer, 2002). On the other hand, dramatic temporal changes in the amino acids were noticed following the pathogen inoculation. Amino acids belonging to five different AAs families were increased in abundances. Different AAs from aspartate, serine and alanine families were found to be activated mainly at the first day after the pathogen inoculation. These AAs loaded significantly to F2, indicating a primary defense response against *P. infestans* attack.

The aspartic acid family of amino acids is produced from the dicarboxylic acid precursor oxaloacetate (OAA) (Fig. 4.2). The plant mainly produces oxaloacetate via two

well studied pathways. The citric acid pathway (Tricarboxylic acid, TCA-cycle) in the mitochondria in which oxaloacetate is produced from the precursor malate with the help of the mitochondrial isoenzyme malate dehydrogenase (EC: 1.1.1.37; KEGG). A second pathway is the production of OAA in the plant cytosol from phosphoenolpyruvate (PEP) with the help of the enzyme PEP carboxylase (EC: 4.1.1.49; KEGG). The OAA produced is then converted to malate with the help of the cytosolic enzyme malate dehydrogenase (EC: 1.1.1.37; KEGG). The dicarboxylic organic acid, fumarate, is the precursor of malate in the TCA cycle. Following pathogen inoculation, the abundances of the two organic acids fumarate (69) and succinate (81) were not increased. Therefore, it is more likely that OAA, the precursor of the aspartic acid, was mainly produced in the plant cytosol. Aspartic acid is the primary block for the production of other AAs of this family. A metabolic pathway that leads to the synthesis of threonine and isoleucine was found to be highly activated in P1, with high loadings to F2 vector for both AAs. Cystathionine (82), a precursor of methionine was neither significantly increased- nor decreased in abundance after the pathogen inoculation and had very low loadings to F2. Thus, in conclusion, the aspartic acid family was mainly increased in abundances in P1, at 1 DAI with P1/W > P2/W >P4/W and had high loadings to F2 vector.

The AAs of the alanine family are produced from the precursor pyruvic acid. As compared to other treatments, the AAs alanine and valine were significantly increased in abundances in P1 with P1/W > P2/W >P4/W and had high loadings to F2. The AAs valine and isoleucine (discussed earlier) have similar metabolic pathways in the chloroplasts and were increased in abundances following the pathogen inoculation. These AAs share four common enzymes during their synthesis namely acetohydroxy acid synthase (EC: 2.2.1.6; KEGG), ketol-acid reductoisomerase (EC: 1.1.1.86; KEGG),

dihydroxy-acid dehydratase (EC: 4.2.1.9; KEGG) and aminotransferase (EC: 2.6.1.42; KEGG) (Fig. 4.2). The AAs valine, Isoleucine and leucine are important in the production of plant defense secondary metabolites like cyanogenic glycosides, and glucosinolates (Coruzzi and Last, 2000). The serine family AAs (L-serine and glycine) are produced from the precursor 3-phosphoglycerate. The AAs Serine had high loadings to F2 and was increased in abundance with P1/W > P2/W > P4/W.

The fatty acid, Stearic acid (58; C18:0) had high factor-loadings to F2 and F3 (P1 and P2). This FA can be desaturated by the enzyme 9-desaturase to produce oleic acid (7; C18:1) which was significantly increased in abundance in P1 and P2, with P1/W > P2/W > P4/W. Oleic acid (7) either undergoes desaturation with the help of the enzyme 12-desaturase to produce linoleic acid (5; C18:2) or hydroxylated with the help of epoxygenase or Cyt P450 enzymes to produce different cutin monomers (http://www.scri.sari.ac.uk/TiPP/pps/Chart.pdf). The FA Linoleic acid (5; C18:2) had high factor-loading to F2 with P1/W > P2/W > P4/W. This FA is also a major precursor of many derivatives of jasmonic acid, a signal molecule involved directly in the defense response against insects and pathogen (Somerville et al., 2000; Weber, 2002).

The glycoalkaloid metabolite, solanidine (11) had high factor-loadings to F2 and F3-scores with P1/W > P2/W > P4/W. This metabolite was reported to have anti-microbial activity (Moehs et al., 1997; Lachman, 2001) and is produced from the cytocylic acetyl-CoA through the mevalonate pathway (Fig. 4.2). Acetyl-CoA in the cytosol can be produced from the organic acid citrate by the enzyme ATP-citrate lyase (Fatland et al., 2005).

4.5.3. Secondary defense phase (F3 = P2):

On the second day after the pathogen inoculation, the aromatic amino acids (tyrosine and phenylalanine) and the glutamate family AAs (glutamine and L-proline) had high loadings to F3, and were highly increased in abundances (P2/W > P1/W > P4/W) indicating a secondary defense response against *P. infestans*.

The OAs α-Ketoglutarate is a precursor of the glutamate family AAs. The conversion of the AA L-Glutamic acid to L-proline (93) involves many steps and intermediates. The AA glutamine (26) was significantly increased in abundance with P2/W > P1/W > P4/W. L-proline is an important precursor in the production of cell wall proteins, i.e. proline-rich proteins (PRPs) and hydroxyproline-rich glycoproteins (HRGPs) (Showalter, 1993). Extensins, a sub-group of HRGPs family, is known for its ability to cross-link and covalently linked to different cell wall components such as pectin. This increases the mechanical strength and rigidity of the plant cell walls (Jackson et al., 2001). Higher amount of the AAs glutamine, arginine and proline (glutamate family AAs) as well as the AAs asparagine and lysine (aspartate family AAs) were found in vitro-grown potato tubers compared to soil-grown tubers (Roessner et al. 2000). Also, an increase in glutamine, glutamate and asparagine amino acids were reported in water stressed tomato leaves (Bauer et al., 1997).

The aromatic AAs L-phenylalanine (35) and L-Tyrosine (31) had the highest loading to F3 and also had the highest P2/W-ratio of metabolic regulation in 2 DAI. The primary precursors of these aromatic AAs are phosphoenolpyruvate and erythrose-4-phosphate that combine together to produce the organic acids shikimate and later chorismate (Fig. 4.2). Two separate pathways are involved in the production of the aromatic AAs. One pathway activates the production of both tyrosine and phenylalanine, and the second pathway produces tryptophan. The enzyme chorismate mutase (EC:

5.4.99.5; KEGG) converts chorismate to prephenate then to phenylalanine and /or tyrosine with the help of different enzymes (Coruzzi and Last, 2000). Tyrosine and phenylalanine are the primary precursors of a wide range of secondary metabolites such as phenolics, coumarines, flavones, isoflavones, isoflavanones, lignins, tannins, and the secondary messenger salicylic acid and many others (Fig. 4.2). These metabolites are very important in the defense response against pathogens and the signal molecules can activate several defense pathways leading to more complex defense (Dixon et al., 2002; Barabasi and Oltvai, 2004). In P2, the abundances of both D-Gluconic acid (19) and Xylulose (42), were increased (Table 4.1). These metabolites are associated with the Pentose Phosphate Pathway (PPP) that supplies the precursor erythrose-4-phosphate that is essential in the aromatic AAs synthesis. Relative to P2, in P1, most of the phosphoenolpyruvate (PEP) was primarily used to produce either pyruvate, the precursor of the alanine family, or OAA, the precursor of the aspartate family. The increased in the abundances of these two families was lower in P2 compared to P1 and that could be the main reason that made PEP more available for the production of the aromatic amino acids. In P2, it appears that the PPP and the aromatic AAs pathways were highly activated.

The abundance of the FA Linolenic acid (C18:3) that loaded highly to W1 (homeostasis) was neither increased nor decreased in P1 but it decreased in P2 and P4. This FA is a potential precursor for the production of the signaling metabolite jasmonic acid and its derivatives, which are known to activate different plant defense responses (Liechti and Farmer, 2002).

The abundance of the FA 7,10,13-Hexadecatrienoic acid (C16:3) that loaded highly to W1 was neither increased nor decreased in P1, but was significantly decreased

in P2 and P4 with P4/W < P2/W < P1/W. Following pathogen inoculation, this FA is auto oxidized to give dinor-oxo-phytodienoic acid (dnOPDA), a potential wound signaling metabolite (Weber et al., 1997; Weber, 2002). The FA 7,10-Hexadecadienoic acid might be a precursor of the FA 7,10,13-Hexadecatrienoic acid and was found also to be significantly decreased in abundance in P2 and P4.

4.5.4. Collapse of the defense (negative loadings to all three factor vectors =P4)

Six metabolites including 2 OAs (97, and 28), 2 FAs (62, and 64), and 2 SRs (83, and 86) had negative loading to F1, F2, F3, and their P/W ratios were: P4/W >P2/W > P1/W. Among the metabolites significantly loaded, only L-Gluconic acid was a PR-metabolite. In the serine, alanine, and aspartate AA families that were activated at 1 DAI, the P4/W ratios were < P2/W < P1/W indicating the reduction in the synthesis of these AAs. In addition, the aromatic and the glutamine AA families that were highly activated at 2 DAI were less activated at 4 DAI and their P4/W ratios were < P1/W < P2/W. Similarly, the OAs production was reduced over time, where the P/W-ratios were P4/W < P2/W < P1/W. The P4/W ratios of FAs were the lowest among treatments. This reduction in the P4/W ratios indicated the collapse of primary and secondary defense responses of the plant.

In this study, the GC/MS technology platform was used for metabolite profiling because of its known sensitivity and selectivity, and also is considered as the most common platform among researchers for studying the plant metabolome (Sumner et al., 2003; Dunn et al., 2005). Several metabolites detected here were either known to have antimicrobial and plant defense properties, or precursors in the pathways of production of those metabolites. Although the number of the metabolites detected and tentatively

identified was small, the results showed the capability of the metabolite profiling and multivariate analyses to identify hidden plant-pathogen interaction functions. The plant primary defense response at 1 DAI was considered to be due to increase in the production of several AAs that belong to Serine, Aspartate, and Alanine families, and different C18 FAs that may be involved in the activation of the jasmonic acid signaling pathways. Activation of these metabolites occurred in different satellites or neurons of network of pathways. On the other hand, at 2 DAI (P2), the pathways activated were different from those in 1 DAI (P1) where glutamate family AAs and the aromatic AAs phenylalanine and tyrosine, the primary blocks of the phenylpropanoid pathway, and the C16 FAs that activate the wound signaling response of the plant were activated. At 4 DAI the primary and the secondary plant defense responses were collapsing and the P4/W ratios were the lowest for the AAs, FAs and OAs. At this stage the plant defense responses were failing and the plant was not able to stop the necrotroph phase of the pathogen that usually takes place in 2-3 DAI (Vleeshouwers et al., 2000). These findings indicate the potential application of metabolic profiling technology and multivariate analyses to identify hidden functions of plant defense and the plausible network of metabolic pathways that produce several metabolites, including some that are known to have antimicrobial activity or lead to their production. Similar studies on different potato breeding lines with varying levels of horizontal resistance or quantitative trait loci (QTLs) against late blight could lead to metabolite phenotyping of cultivars to high throughput screen for disease resistance.

4.6. Acknowledgements

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PREFACE TO CHAPTER 5

Chapter 5 is comprised of a manuscript by myself, Dr. A. C. Kushalappa, Dr. S. O. Prasher, Dr. K. Al-Mughrabi, and Ms. A. Murphy. The manuscript has been submitted to "Phytopathology- an International Journal of the American Phytopathological Society" very soon. The contributions of the co-authors have been described in "Contributions to Authors" section. All literature cited has been placed at the end of the thesis.

In the previous chapter the resistance related metabolites were better detected at 48 h, accordingly 48 h incubation was selected for further metabolic profiling work. Though testing both 24 and 48 h would be better, we had to reduce to one incubation time because of the volume of data generated and the time taken to process.

This study reports evaluation of horizontal resistance in 9 cultivars/lines of which 6 cultivars are widely grown in Canada and 2 breeding lines (F86021 and F90008) and 1 cultivar (Libertas) that are considered as potential lines/cultivars to be included in the breeding programs in Canada (Agnes Murphy, Personal Communication). These cultivars are known to have different levels of resistance to *P. infestans,* A1 mating type. The cultivars/lines were first evaluated for the resistance against *P. infestans* using the isolate 1661 that belong to the A2 mating type. Three cultivars with varying levels of horizontal resistance were selected for metabolic profiling. The three cultivars inoculated with water or pathogen and samples injected in GC/MS using protocols developed by Fiehn et al., (2000a,b). A total of 94 metabolites were detected in this study compared to 106 metabolites in the second study. Some metabolites such as á-Sitosterol, Stigmasterol, and Solanidine that eluted at high temperatures were difficult to be quantified in this study because of the high column bleeding. The abundances of the 94 metabolites

identified here were subjected to ANOVA to identify the pathogenesis Related (PR) and the Defense Related (DR) -metabolites. 60 PR-metabolites were further analyzed using FACTOR analysis to identify biological functions. The factor scores were used to classify treatments. The sets of metabolites with significant loadings were used to explain the plausible biological functions, including putative metabolic pathways activated by the resistant cultivars following pathogen inoculation. Several DR-metabolites including phenylalanine, shikimic acid and malonic acid were found to have the highest abundances after the pathogen inoculation of the most resistant cultivar, Libertas. These metabolites are precursors for many plant defense and antimicrobial compounds.

CHAPTER 5

Metabolic profiling to phenotype potato cultivars varying in horizontal resistance to leaf infection by *Phytophthora infestans*.

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5.1. Abstract

Screening potato breeding lines, based on disease severity, for horizontal resistance to *Phytophthora infestans* is slow and results are inconsistent over the years. This study reports the potential use of metabolic phenotyping as an additional or alternative tool for screening potato lines/cultivars for disease resistance. Three potato cultivars Libertas, Caesar and Russet Burbank with high, moderate and low resistance to *P. infestans*, respectively, were inoculated with the pathogen or water, and metabolites were analyzed using GC/MS. Ninety-four metabolites were tentatively identified of which 60 metabolites were identified as pathogenesis related (PR) metabolites. FACTOR analysis of PR-metabolites identified metabolic phenotypes similar to disease severity phenotypes. The first three factors explained 74. 50% of the total variance, and the F1, F2 and F3-vectors identified the resistance, moderate resistance and susceptibility functions, respectively. Since the three factors were orthogonal and independent of each other, the results imply that the regulation of metabolites in response to the pathogen attack were unique to a cultivar. Thirty metabolites had high loadings to F1-vector that discriminated the high resistance in cv. Libertas. These metabolites were considered as Defense Related Metabolites (DR-metabolites). 25 of these metabolites belonged to organic acids and amino acids groups. Some of these metabolites i.e. phenylalanine, tyrosine, shikimic acid and malonic acid were highly increased in abundances. These metabolites are known for their ability to activate plant secondary defense metabolism particularly the Phenylpropanoid and Malonic acid pathways. 26 of the 30 DR-metabolites were common to Caesar and Libertas and were considered as plant Resistance Specific Defense Related metabolites (RSDR-metabolites). Using the metabolic profiling approach we were able to discriminate three potato cultivars with

different levels of the horizontal resistance to *P. infestans* and also to relate resistance functions to specific groups of metabolites and putative pathways that activate the production of antimicrobial compounds.

Abbreviations: GC/MS = Gas Chromatography/Mass Spectrometry; PR-metabolites = Pathogenesis Related metabolites; DR-metabolites = Defense Related metabolites; GDR-metabolites = General Defense Related Metabolites; RSDR-metabolites = Resistance Specific Defense Related metabolites.

Additional key words: Factor analysis, GC/MS, Horizontal Resistance, Metabolomics, *P. infestans*, PR-metabolites, *Solanum tuberosum*.

5.2. Introduction

Late blight caused by *Phytophthora infestans* (Mont.) de Bary is one of the major diseases of potato (*Solanum tuberosum*) in Canada, particularly after the appearance of A2 mating type (Goodwin et al., 1998; Peters et al., 1998; Daayf et al., 2000; Daayf and Platt, 2002). This aggressive mating type can sexually interbreed with the A1 mating type to produce oospores and causes the appearance of new races (Fry et al., 1993; Daayf and Platt, 1999; Peters et al., 1999; Daayf et al., 2000; Stromberg et al., 2001). In spite of the high cost and harmful effects to the environment, fungicides have been extensively used to control this disease. The annual worldwide losses caused by *P. infestans* including the cost of control measures exceed US$ 3 billion (Duncan, 1999). Breeding for the vertical resistance has been the most versatile method to control this pathogen. Unfortunately, this type of resistance is not durable and can be broken down easily by emergence of new races of this pathogen. On the contrary, horizontal resistance is considered to be more durable in the field (Peters et al., 1999; Haynes et al. 2002). Although high levels of horizontal resistance have been detected in non-cultivated and wild Solanum species, the progress made in transferring horizontal resistance to cultivated potatoes has been very limited because of the difficulty in breeding for polygenic traits (Evers et al., 2003). Multiple epidemiological disease parameters such as infection efficiency, latent period, lesion expansion, sporulation, etc. can separate quantitative resistance (Carlisle et al., 2002), but these tests are time consuming and expensive for use in breeding programs. Plant breeders are looking for tools to phenotype cultivars varying in quantitative resistance and also to better understand the mechanism of resistance. Metabolic phenotyping of cultivars varying in resistance to disease may be a potential alternative.

Metabolic profiling and fingerprinting have been used extensively to study genetically modified traits (Roessner et al. 2001a,b), single gene mutants of *Arabidopsis thaliana* (Fiehn et al, 2000b), salt-stressed tomato (Johnson et al., 2003), primary metabolism in rice leaves (Sato et al., 2004), transgenic *Nicotiana tabacum* (Mungur et al., 2005), biotic and abiotic stressed *Medicago truncatula* (Broeckling et al, 2005), comparing the level of metabolites in wild and cultivated tomatoes (Schauer et al., 2005), wheat cultivars with different levels of resistance to fusarium head blight (Hamzehzarghani et al., 2005), discriminating wild-type and transgenic lines of *Populus* spp. (Robinson et al., 2005), and metabolic profiling of *Arabidopsis thaliana* leaves (Kolbe et al., 2006). The main objectives of this study were to discriminate potato cultivars varying in the horizontal resistance to *P. infestans* using the metabolic profiling technique and to classify the DR-metabolites associated with the most resistant cultivars. Based on this, a technology can be developed for metabolic phenotyping of resistance in potato against late blight.

5.3. Materials and methods

5.3.1. Potato plant production

Elite seed tubers of nine potato cultivars/lines: AC Brador, AC Novachip, Caesar, Dorita, F86021, F90008, Libertas, Russet Burbank, and Shepody were obtained from the Potato Research Center, Agriculture and Agri Food Canada, New Brunswick. Tubers were planted in 16 cm diameter pots (one tuber per pot) containing mixture of 1:1 ratio of soil and PRO-Mix BX® (Premier Horticulture Ltd, Riviere-du-Loup, QC) and maintained at 20 °C, 16 h photoperiod and around 70% relative humidity in a growth bench. Plants

were fertilized weekly with 200 ml pot^{-1} of a solution (1.5g per L) of Plant-Prod® 20:20:20 containing trace elements (Plant Products Co. Ltd., Ontario, Canada). One to three stems per plant/pot were maintained.

5.3.2. Pathogen

Phytophthora infestans (Mont.) de Bary (clonal lineage US-8, A2 mating type, isolate 1661) was obtained from AAFC, Charlottetown, PEI. The pathogen was sub-cultured on V-8-Agar media (Caten and Jinks, 1968) at 15 °C. After 2-3 weeks a sporangial suspension was prepared using sterilized water containing 0.02% Tween 80. The sporangial concentration was adjusted to 1.0×10^5 sporangia ml^{-1}.

5.3.3. Inoculation and incubation

Either the detached or the in-plant leaves were inoculated. For the detached leaf inoculation, the fully-grown leaflets were detached, placed on plastic benches lined with moist paper towels and placed in plastic tray-incubators with transparent covers (28 x 54 cm). Water was added to the bottom of the trays to maintain high humidity. The leaflets were inoculated with 5 µl of the sporangial suspension. The trays were kept in a growth chamber at 18 °C and 90% RH.

For the in-plant leaf inoculation, three days before inoculation, 5-6 week old plants grown in a growth bench were transferred to a growth chamber maintained at 20 °C, 16 h photoperiod and 90% relative humidity. The fully developed leaflets were inoculated on their lower surface, at either sides of the midrib, with the sporangial suspension or mock (aqueous solution of Tween). The plants were misted with sterile

water, covered with transparent plastic bags to maintain high humidity, and returned to the growth chamber. The bags were removed 48 h after inoculation.

5.3.4. Disease severity and sporulation assessment

At 4 and 6 d after inoculation (DAI) of leaf discs, the diameters of lesions were measured, from which lesion areas were calculated.

5.3.5. Metabolite extraction

At 48 h after inoculation (HAI) of in-plant leaflets, discs containing the inoculated lesions were cut using a 15 mm cork borer, frozen in liquid nitrogen, lyophilized for 48 h and stored at –80 °C until extraction. The polar and non-polar metabolites were extracted following methods developed by Fiehn et al. (2000 a, b) with minor modifications. The lyophilized discs were crushed in liquid nitrogen and a 15.00 ± 0.01 mg sample was used for metabolites extraction. 0.70 ml methanol and 175 µl double distilled water were added and vortexed to which 25 µl Ribitol (0.2 mg ml^{-1} of water) and 50 µl Nonadecanoic acid methyl ester (2 mg ml^{-1} of chloroform) were added as internal standards. The sample was heated at 70 °C for 15 min, centrifuged for 3 min at 13,500 rpm and the supernatant was transferred to a glass tube provided with a screw cap with teflonized inlays, to which 0.70 ml double distilled water was added. For the remaining pellets, 0.75 ml chloroform was added, vortex, heated at 37 °C for 5 minutes with continuous shaking and centrifuged for 3 min at 13,500 rpm. The supernatant chloroform portion (non-polar) and the previously obtained water/methanol portion (polar) were

transferred to a 15 ml Millipore Ultrafree®-CL Filters (Amicon, Bioseperation) tube, vortexed and centrifuged for 15 min at 3,800 rpm.

The upper polar phase was separated, dried using a Speed Vac (SAVANT DNA110, Thermo Electron Co.), 60 µl methoxyamine hydrochloride (20 mg ml^{-1} pyridine) was added, heated at 30 °C for 90 min and derivatized with 96 µl MSTFA and heated for 30 min at 37 °C. 40 µl of the sample was transferred to GC screw top amber glass vial, 25 µl each of the three (Naphthalene, Phenanthrene and Chrysene) Lee's retention time index standards (Eckel, 2000) were added and the end volume of the sample was adjusted to 1 ml by adding 885 µl of hexane. 1 µl of the sample was injected into GC/MS in splitless mode.

The non-polar chloroform-phase was transferred to a new vial and 0.90 ml of chloroform and 1 ml of methanol containing 3% v/v H_2SO_4 was added. Sample was heated for 4 h at 100 °C to transmethylate lipids and free fatty acids. Each sample was cleaned twice by adding 4 ml of pure water, vortexed and centrifuged for 15 minutes at 3,800 rpm. The water phase was discarded and anhydrous sodium sulphate was added to the remaining non-polar extract to remove excess of water. The supernatant was dried using a Speed Vac, 80 µl of chloroform was added and derivatized with 10 µl MSTFA and 10 µl pyridine at 37 °C for 30 min. 33 µl of the end sample was transferred to GC screw top amber glass vial, 25 µl of each of the three Lee's retention time index standards were added and the end volume was adjusted to 1 ml by adding 892 µl of hexane. 1 µl of the sample was injected into the GC/MS in splitless mode.

5.3.6. GC/MS analysis

The leaf extract samples in vials were transferred to an auto sampler (model 8200 CX, Varian®, Canada) connected to a GC/MS (GC Varian®, Saturn 3400 CX with MS 2000, Varian ® Saturn, Canada). The GC was equipped with a capillary column (30 m DB-5MS column with 0.25 mm diameter, 0.25 μm film thickness, Supelco, Canada). The initial injector temperature was 230 °C. Helium was used as a carrier gas with a flow rate of 1 ml s^{-1}. For the methanol-water samples, the initial oven temperature was programmed at 70 °C for 5 min and then increased at a rate of 4 °C min^{-1} to 280 °C then increased at a rate of 20 °C min^{-1} until 290 °C and held for 5 min. For the chloroform extract the ramping temperature was 5 °C min^{-1} from 70 °C to 290 °C. The mass spectra from 50 to 600 m/z were recorded using ion trap analyzer. The GC/MS outputs on scans and abundances of mass ions were obtained using Saturn Lab Software Version 5.52.

5.3.7. Mass spectral data processing

The GC/MS output on abundances of mass ions at different scans were imported into a spreadsheet and organized using the Pivot Table operation of the EXCEL® program. The abundances of peaks were corrected for each sample with reference to the internal standards, Ribitol for the water-methanol samples and Nonadecanoic acid methyl ester for the chloroform samples. The mass ion spectra of peaks with about the same retention time in five replicates of each treatment was inspected using SATURN workstation version 5.52 and the most probable choice of a name was selected for the compound using NIST Library Version 2.0 (National Institute of Standards and Technology, MD, USA). In addition, the spectra were further compared with Golm

Metabolome Database (http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/gmd.html) (Kopka et al., 2005). The low probability matched peaks were considered as unidentified and their spectra (m/z) in decreasing order of relative abundance were given in place of names. Metabolites that were not consistent among replicates were excluded from the analysis.

The Lee Retention Index (RI) was calculated for the metabolites (Lee et al., 1979; Eckel, 2000): $RI = \{100 \times (RT_{Unknown} - RT_n)/ (RT_{n+1} - RT_n)\} + 100 (n)$, where $RT_{unknown}$ is the Retention Time of the unknown metabolite; the RT_n and RT_{n+1} are the retention time of the standards that eluted before and after the unknown, respectively. The number of rings of the aromatic standards represents (n), i.e. n=2 for naphthalene, n=3 for phenanthrene and n=4 for chrysene. The Lee RI was also used to confirm or reject the metabolites names proposed by search libraries (NIST and Golm) based on the boiling point in degrees Celsius, where the metabolites with boiling points (obtained from SciFinder® Scholar Version 2002, American Chemical Society) less than their RI were considered as miss identified (Eckel, 2000) and re designated as unidentified. For some of the tentatively identified metabolites, the total abundances abnormally varied among the 5 replicates. Manual integration, using Chromatogram Integration Dialog Box of Saturn View ™ Version 5.52, of each of the peaks in the 5 replicates was carried out by integrating the total abundance of the top 10 m/z ions of the proposed name of the prospective metabolite.

5.3.8. Experimental design

Two experiments were conducted: i) Disease severity assessment: The experiment was designed as completely randomized with nine cultivars (names given

above) inoculated with the pathogen and five replicates. The experimental units consisted of 15 inoculated leaflet-discs selected from three different plants. The data on disease severity (average lesion area in mm^2) were subjected to GLM using SAS program and Student-Newman-Keuls (SNK) multiple range tests at (P=0.05) was used to compare the means of different treatments. ii) Metabolite profiling: The experiment was a factorial with two factors consisting of three cultivars (Libertas, Caesar and Russet Burbank) and two inoculations (pathogen and water) designed as a completely randomized block. The blocks were conducted 5 times (about weekly intervals). Each experimental unit consisted of 20 leaflet discs cut from two plants. The data on abundances of 94 metabolites were subjected to ANOVA using GLM procedure of SAS to identify the compounds significantly different among treatments by using the Student-Newman-Keuls (SNK) test at P=0.05 level. Sixty metabolites significantly varying in abundances between pathogen and water inoculations in at least in one cultivar were found and designated as pathogenesis related (PR) metabolites. These metabolites were further subjected to FACTOR analysis, using principal components and the orthogonal (Orthomax) rotational methods of SAS, to classify the treatments. The factor-vectors that classified the treatments were used to identify the hidden interaction functions by relating the treatments within a class to resistance based on disease severity. The functions were explained using the set of metabolites highly loading (positively or negatively) to that factor-vector.

5.4. Results

5.4.1. Disease severity

The average lesion areas at 4, and 6 d after inoculation (DAI) for the 9 cultivars are presented in Table 5.1. The cultivars varied in their disease severity or levels of resistance. Based on the disease severity the cultivars were arbitrarily classified into three groups: i) highly resistant: Libertas and F86021; ii) moderately resistant: Caesar, Dorita, and AC Brador; and iii) susceptible: Russet Burbank and F90008.

5.4.2. Potato-*Phytophthora* interaction metabolic profiles

More than 300 peaks were detected, of which only 94 metabolites with abundances >2,000 and consistent in all the 5 replicates were tentatively identified (Table 5.1). Out of 94 metabolites, 60 were PR-metabolites (metabolites that varied significantly, up or down, in their abundances between pathogen and water-inoculated in at least one of the tested cultivars). 42, 46 and 22 PR-metabolites were found in Libertas, Caesar and Russet Burbank, respectively.

Among the 42 Libertas PR-metabolites, 15 were organic acids (OAs) including: [1, 2, 5, 8, 13, 14, 19, 24, 28, 30, 39, 45, 46, 58, and 74]; 12 amino acids (AAs) including: [4, 6, 9, 10, 11, 15, 18, 20, 25, 31, 32, and 54]; 7 fatty acids (FAs) including: [78, 79, 80, 85, 86, 87, and 92]; 4 sugars (SRs) including: [29, 36, 50, and 72]; 3 Phenolics including: [60, 62, and 67] and 1 unidentified compound [47]. Among these PR-metabolites, 30 metabolites [1, 2, 4, 5, 6, 8, 9, 10, 11, 13, 14, 15, 18, 19, 20, 24, 25, 29, 30, 31, 32, 36, 45, 46, 54, 58, 62, 67, 74, and 92] were significantly increased in abundances in Pathogen-inoculated (PI) Libertas, the most resistant cultivar, and were significantly different from the rest of the treatments. These metabolites are considered as Defense Related Metabolites (DR-metabolites; Fig. 5.2A-C). The same metabolites had the highest score loadings for the High Resistance Function (F1) as we will explore

in the next section. Nine of the 30 DR-metabolites [1, 2, 4, 5, 18, 29, 36, 46 and 58] were significantly increased in abundances after the pathogen inoculation in the three tested cultivars and considered as General Defense Related metabolites (GDR-metabolites; Fig. 5.2 A). Although these metabolites were increased in abundances in the three cultivars after the pathogen inoculation, the highest increase was reported in Libertas. All the 30 DR-metabolites except for four [8, 11, 13, 92] were increased in abundances in both Libertas and Caesar (the moderately and the highly resistant cultivars) and considered as plant Resistance Specific Defense Related metabolites (RSDR-metabolites) (Table 5.2; Fig. 5.2B). Although these metabolites were increased in abundances in both cultivars, the highest abundances were found in Libertas. Taking a comprehensive look to ANOVA results, 13 of the 30 DR-metabolites were found to have the highest abundances in both the water-inoculated and the pathogen-inoculated Libertas when compared to the other two cultivars, including: [2, 5, 6, 9,13, 14, 19, 31, 32, 45, 62, 67, and 92]. Therefore, these metabolites are considered as Resistance Constitutive Defense Related metabolites (RCDR-metabolites; Fig. 5.2C) and they could be viewed as plant constitutive markers for the high level of resistance.

Among the 46 Caesar PR-metabolites, 16 were OAs including: 1, 2, 3, 5, 14, 16, 19, 24, 26, 28, 30, 39, 45, 46, 58 and 74; 11 AAs including: 4, 6, 9, 10, 15, 18, 20, 25, 31, 32 and 54; 8 FAs including: 75, 78, 80, 83, 84, 85, 86, and 88; 5 SRs including: 23, 29, 36, 50 and 51; 3 Phenolics including: 60, 62 and 67; 3 to other groups including; 76, 77, and 90.

Among the 22 Russet Burbank PR-metabolites, 9 were OAs including; [1, 2, 3, 5, 13, 26, 41, 46 and 58]; 6 SRs including: [22, 23, 29, 36, 63 and 71]; 3 FAs including: [79, 87 and 91]; 2 AAs [4 and 18]; 2 unidentified [61 and 90].

5.4.3. Potato-*Phytophthora* interaction functions and the related metabolites

Given that our main interest in this study was to find, discriminate and to establish correlations among the PR-metabolites of the different cultivars, the 60 PR-metabolites were considered in FACTOR analysis of SAS to classify cultivars and to identify hidden functions. The abundances of 60 PR-metabolites were subjected to factor analysis to classify the six treatments. The first three factors explained 74% of the total variance, with 38%, 20%, and 16% by F1, F2, and F3, respectively (Table 5.2). The factor analysis classified the replicates of six treatments into six different groups, meaning the variance among replicates were minimal. The pathogen and water inoculated treatments were clustered closely in all the cultivars, still keeping the cultivars in separate clusters. The pattern of classification of the treatments was then related to the resistance of cultivars to late blight (based on disease severity, Table 5.1) to identify the hidden host-pathogen interaction functions. The F1, F2 and F3 vectors identified three functions: high resistance, moderate resistance and pathogenesis functions, respectively (Fig. 5.1).

5.4.3.1. High resistance function

F1-vector explained 38% of the variance and it classified the most resistant cv. Libertas (high positive F1-scores) from rest, the moderately resistant and susceptible cultivars (negative F1-scores); meaning the set of PR-metabolites with high positive loadings to F1-vector explained high level of resistance in Libertas, a cultivar which had the highest resistance based on disease severity. Thirty metabolites with high (>0.5) positive loadings to F1 were: 32, 6, 9, 5, 45, 31, 62, 13, 14, 2, 24, 19, 8, 18, 92, 30, 54,

25, 10, 20, 67, 74, 58, 1, 15, 4, 36, 29, 11 and 46 (metabolite numbers in descending order, details in Table 5.2). According to ANOVA analysis, these DR-metabolites had the highest abundances in (PI) Libertas, the most resistant cv. compared to other treatments. Therefore, these metabolites will be the main focus of this study (Fig. 5.2 A-C). Among these metabolites, thirteen were organic acids (OAs), 12 amino acid (AAs), 2 sugars (SRs), 1 fatty acid (FA), and 2 belonged to other groups-phenolics (OP). Among the 13 OAs that increased in abundances, four were members or derivatives of the Citric Acid Cycle including 2-Butenedioic acid (14= Fumaric acid; Fig. 5.2 C2), Butanedioic acid (24= Malic acid), Butanedioic acid [30], and 1,2,3-Propanetricarboxylic acid (46= Isocitric acid). Other organic acids, (2= methyl malonate, Malonic acid derivative), Propanedioic acid (5= Malonic acid; Fig. 5.2 C1), Benzoic acid [74], and 1-Cyclohexene-1-carboxylic (45=Shikimic acid; Fig. 5.2 B2) are primary blocks involved in the production of many plant defense secondary metabolites and were found to be highly increased in abundances. Of the 12 AAs that increased in abundances, two belonged to the Alanine family including L-Alanine [4] and L-Valine [6]; Three to the Serine family including: Glycine [11 and 20], and L-Serine [15]; Two to the Aromatic AAs including L-phenylalanine [32; Fig. 5.2 B1] and L-tyrosine [54]; Three to Aspartic acid family including: L-Isoleucine [9], L-threonine [18; Fig. 5.2 A1] and L-Aspartic acid [25] and 2 belong to Glutamine family including L-Proline [10], and Glutamine [31]. The two SRs that increased in abundances were Xylulose [29; Fig. 5.2 A2] and d-Ribose [36].

5.4.3.2. Moderate resistance function

F2-vector explained 20% of the variance in the abundances of 60 metabolites and classified the moderately resistant cultivar Caesar (high positive F2-scores), from the highly resistant cultivar Libertas (low positive and negative F2-scores and the susceptible cultivar Russet Burbank (high negative F2-scores). Ten metabolites had high (>0.5) positive loading to F2 and these were: 28, 16, 3, 72, 46, 60, 4, 47, 29, and 67 (descending order, Table 5.2). Four of these metabolites were OAs, 2 SRs, 2 Phenolics, 1 AAs and 1unidentified. All of these were PR-metabolites in Caesar. Four of these metabolites [3, 16, 28, and 60] had the highest up-regulation levels in PI Caesar.

5.4.3.3. Susceptibility function

F3-vector explained 16% of the variance in the abundances of 60 metabolites and classified the susceptible cultivar Russet Burbank from the others. The susceptible cv. Russet Burbank had the highest F3-scores and the all the cultivars and as well as the pathogen inoculation treatments from water inoculations. The F3-scores were higher for the pathogen than the water inoculated, especially for the resistant cultivars. 14 metabolites with high (>0.5) loading to F3 were: 39, 85, 88, 84, 22, 75, 83, 15, 86, 90, 23, 41, 58, and 36 (descending order, Table 5.2). Of these PR-metabolites 5 were increased in abundances and 1 decreased in the pathogen-inoculated Russet Burbank.

Table 5.1. Disease severity[1], based on detached leaflets, of 9 potato cultivars/lines inoculated with *P. infestans*. The lesion areas (mm^2) were measured 4 and 6 days after inoculation.

Cultivar	Day 4	Day 6
Russet Burbank	130.99^{A}	143.92^{A}
F90008	120.30^{AB}	145.41^{A}
AC Novachip	103.63^{BC}	121.75^{B}
Shepody	88.25^{CD}	110.07^{B}
AC Brador	79.99^{DE}	110.58^{B}
Dorita	76.14^{DE}	82.40^{C}
Caesar	64.24^{EF}	87.32^{C}
F86021	54.13^{FG}	72.27^{CD}
Libertas	37.99^{G}	57.54^{D}

The disease severity was measured as average area in mm^2 and was analyzed using GLM of SAS and the means were compared using Student-Newman-Keuls (SNK) test at $P \leq .05$.

Table 5.2. Metabolites and their abundances ($x10^6$) detected in leaves of 3 potato cultivars[1] Caesar, Libertas, and Russet Burbank inoculated with *P. infestans* or water (control) and the factor loading of metabolites to factor vectors based on factor analysis of 60 PR-metabolites.

NO.[2]	RI[3]	Name[4]	CAS No.[5]	GR[6]	CW	C P/W	LW	L P/W	RW	R P/W	F1[7]	F2	F3
6	203.9	L-Valine, N-(trimethylsilyl- (N,G)	7364-44-5	AA	0.29^{E}	1.77^{B}	0.45^{C}	1.964^{A}	0.34^{D}	1.14^{D}	0.95	0.23	0.12
32	274.1	L-phenylalanine (N)	7364-51-4	AA	0.15^{D}	1.73^{C}	0.35^{B}	2.248^{A}	0.14^{D}	1.21^{D}	0.95	0.13	-0.03
9	218.6	L-Isoleucine, N-(trimethy- (G,N)	7483-92-3	AA	0.22^{D}	1.85^{B}	0.37^{B}	2.355^{A}	0.27^{CD}	1.25^{BC}	0.94	0.22	0.14
45	301.0	Shikimic acid- (G,N)	55520-78-0	OA	1.25^{C}	1.28^{B}	1.54^{B}	2.221^{A}	1.31^{C}	1.11^{BC}	0.93	0.19	0.14
5	202.3	Propanedioic acid, bis(tri- (N,G)	18457-04-0	OA	0.28^{D}	1.14^{C}	0.41^{B}	1.283^{A}	0.24^{E}	1.19^{D}	0.93	0.04	-0.21
31	273.4	Glutamine, tris(trimethyls- (G,N)	15985-07-6	AA	3.28^{D}	1.55^{B}	4.06^{C}	2.240^{A}	3.16^{D}	1.12^{D}	0.92	0.33	0.08
62	341.7	Silanamine, N-[2-[3,4-bis[(tr- (N)	55606-74-1	O(P)	0.03^{D}	4.65^{C}	0.25^{B}	1.355^{A}	0.04^{D}	1.83^{D}	0.92	-0.02	-0.23
13	225.4	Propanoic acid, 2,3-bis[(t- (G,N)	38191-87-6	OA	2.03^{C}	1.02^{C}	3.72^{B}	1.239^{A}	1.56^{D}	1.27^{C}	0.91	-0.12	-0.3
2	175.1	Methyl 2-(trimethylsilyl)ethyl malonate (N)	N/A	OA	0.08^{C}	1.28^{B}	0.10^{B}	1.295^{A}	0.06^{D}	1.26^{C}	0.88	0.29	-0.19
14	229.7	2-Butenedioic acid (E)-, - (G,N)	17962-03-7	OA	0.13^{D}	1.23^{C}	0.23^{B}	1.168^{A}	0.09^{E}	1.11^{E}	0.88	-0.04	-0.41
19	239.8	Butanoic acid, 2,4-bis[(trim- (N)	55191-52-1	OA	0.03^{D}	1.41^{C}	0.05^{B}	1.190^{A}	0.02^{E}	1.12^{E}	0.82	0.16	-0.47
24	252.9	Butanedioic acid, [(trime- (N,G)	38166-11-9	OA	24.01^{D}	1.09^{C}	29.07^{B}	1.136^{A}	27.39^{BC}	1.04^{B}	0.82	-0.22	0.26
8	215.0	Silanol, trimethyl-, phosp- (N,G)	10497-05-9	OA	3.71^{B}	1.15^{B}	4.13^{B}	1.348^{A}	3.50^{B}	1.00^{B}	0.81	0.27	-0.09

18	235.1	L-threonine (N,G)	7537-02-2	AA	0.52^{D}	1.66^{B}	0.55^{D}	2.271^{A}	0.59^{D}	1.22^{C}	0.80	0.38	0.31
92	364.9	Octadecanoic acid, trim- (N,G)	18748-91-9	FA	0.43^{D}	1.00^{D}	0.67^{B}	1.183^{A}	0.56^{C}	1.09^{BC}	0.79	-0.48	0.14
30	269.6	Butanedioic acid, 2,3-bis- (G,N)	38165-94-5	OA	0.05^{C}	1.28^{B}	0.045^{C}	1.607^{A}	0.05^{C}	1.06^{C}	0.78	0.48	0.13
25	257.8	L-Aspartic acid, N-(trimet- (G,N)	55268-53-6	AA	3.09^{C}	1.44^{B}	2.99^{C}	1.979^{A}	3.21^{C}	1.13^{C}	0.78	0.44	0.27
54	321.4	L-Tyrosine, N,O-bis(trim- (N,G)	51220-73-6	AA	0.12^{C}	1.89^{B}	0.11^{C}	3.359^{A}	0.11^{C}	1.47^{C}	0.78	0.44	0.21
10	219.2	L-Proline, 1-(trimethylsilyl- (G,N)	7364-47-8	AA	0.10^{C}	1.54^{B}	0.10^{C}	1.919^{A}	0.10^{C}	1.23^{C}	0.77	0.40	0.22
20	242.7	Glycine, N,N-bis(trimethy- (N,G)	25688-73-7	AA	0.50^{CD}	1.16^{B}	0.56^{BC}	1.178^{A}	0.43^{D}	1.06^{D}	0.76	0.35	-0.3
67	352.6	Norepinephrine, N,N,O,O- (N,G)	56114-59-1	O (P)	0.15^{D}	2.83^{B}	0.22^{C}	2.294^{A}	0.07^{E}	1.64^{DE}	0.73	0.56	-0.15
74	232.2	Benzoic acid, 2-methyl-, tri- (N)	55557-15-8	OA	0.03^{C}	1.78^{B}	0.03^{C}	2.082^{A}	0.04^{C}	1.15^{C}	0.70	0.39	0.32
58	331.3	D-Gluconic acid, 2,3,4,5- (N,G)	34290-52-3	OA	0.07^{D}	1.22^{C}	0.09^{C}	1.583^{A}	0.10^{C}	1.26^{B}	0.67	-0.10	0.57
1	173.1	Propanoic acid, 2-[(trim- (N,G)	17596-96-2	OA	0.09^{C}	1.29^{A}	0.10^{C}	1.297^{A}	0.09^{C}	1.17^{B}	0.66	0.47	0.28
15	230.8	L-Serine, N,O-bis(trimet- (G,N)	7364-48-9	AA	0.92^{D}	1.39^{B}	1.02^{C}	1.582^{A}	1.28^{B}	1.06^{B}	0.64	0.16	0.70
4	181.8	l-Alanine, N-(trimethylsily- (G,N)	27844-07-1	AA	0.36^{C}	1.18^{B}	0.28^{E}	1.790^{A}	0.24^{F}	1.31^{D}	0.63	0.73	-0.03
29	266.9	Xylulose tetra-TMS (G,N)	N/A	SR	0.83^{B}	1.19^{A}	0.80^{BC}	1.288^{A}	0.72^{C}	1.18^{B}	0.59	0.66	0.07
36	281.4	d-Ribose, 2,3,4,5-tetrakis- (N,G)	56196-08-8	SR	0.35^{D}	1.44^{C}	0.60^{B}	1.218^{A}	0.56^{BC}	1.33^{A}	0.59	-0.38	0.54
11	220.6	Glycine, N,N-bis(trimethy- (G,N)	5630-82-0	AA	0.11^{B}	1.07^{B}	0.11^{B}	1.452^{A}	0.12^{B}	1.11^{AB}	0.55	0.06	0.37
46	301.9	1,2,3-Propanetricarboxylic- (N)	14330-97-3	OA	6.44^{C}	1.12^{B}	5.18^{E}	1.517^{A}	5.22^{E}	1.12^{D}	0.53	0.79	0.07

86	344.8	9,12-Octadecadienoic aci- (N,G)	112-63-0	FA	3.45^{C}	1.13^{B}	4.05^{B}	1.107^{A}	4.41^{A}	1.03^{A}	0.39	-0.39	0.69
47	307.6	Unkown (345,346,73,255,347)	N/A	N/A	11.92^{A}	1.05^{A}	9.45^{B}	1.222^{A}	4.68^{C}	1.09^{C}	0.32	0.69	-0.62
71	384.5	Mannose, 6-deoxy-2,3,4,5-t - (N)	19127-15-2	SR	0.16^{AB}	1.23^{A}	0.16^{AB}	1.142^{AB}	0.13^{B}	1.40^{A}	0.26	0.36	0.03
60	333.9	Silanamine, 1,1,1-trimet- (N,G)	55556-99-5	O (P)	0.15^{B}	1.41^{A}	0.12^{C}	1.369^{B}	0.11^{C}	0.87^{C}	0.23	0.78	-0.26
26	259.2	Butanoic acid, 4-[bis(trim- (G,N)	39508-23-1	OA	1.43^{B}	1.10^{A}	1.43^{B}	1.062^{AB}	1.26^{C}	1.24^{A}	0.23	0.42	0.09
85	340.4	9-Octadecenoic acid (Z)-, m- (N)	112-62-9	FA	0.02^{C}	1.34^{B}	0.02^{C}	1.867^{A}	0.04^{A}	1.12^{A}	0.21	0.05	0.84
72	413.0	à-D-Glucopyranoside, 1,3,4- (N)	19159-25-2	SR	18.74^{A}	1.10^{A}	13.49^{B}	1.352^{A}	11.57^{B}	0.94^{B}	0.20	0.81	-0.36
28	263.9	2,3,4-Trihydroxybutyric acid- (N)	38191-88-7	OA	2.19^{B}	1.17^{A}	1.41^{C}	1.429^{B}	1.10^{C}	1.00^{C}	0.14	0.86	-0.37
84	337.0	Hexadecanoic acid, trim- (G,N)	55520-89-3	FA	0.30^{C}	1.59^{B}	0.46^{BC}	1.34^{AB}	0.76^{A}	1.04^{A}	0.13	-0.38	0.72
23	251.2	Arabino-Hexos-2-ulose, 3,4- (N)	74685-71-5	SR	0.37^{C}	1.11^{B}	0.41^{BC}	1.121^{B}	0.46^{B}	1.20^{A}	0.09	-0.21	0.64
63	342.7	Inositol, 1,2,3,4,5,6-hexakis- (N)	14251-18-4	SR	7.04^{A}	1.07^{A}	8.73^{A}	0.863^{A}	8.41^{A}	0.76^{B}	0.09	-0.25	-0.25
22	249.4	Xylo-hexos-5-ulose, 2,3,4,6- (N)	62108-13-8	SR	0.18^{E}	1.07^{DE}	0.25^{CD}	1.157^{BC}	0.32^{B}	1.27^{A}	0.08	-0.51	0.71
87	345.9	9,12,15-Octadecatrienoic- (N,G)	301-00-8	FA	8.02^{D}	1.02^{D}	10.62^{AB}	0.881^{C}	10.95^{A}	0.93^{B}	0.08	-0.84	0.26
76	306.4	3,7,11,15-Tetramethyl-2- (1) (N)	102608-53-7	O(AA)	0.32^{B}	1.36^{A}	0.44^{A}	0.896^{AB}	0.46^{A}	0.97^{A}	0.01	-0.41	0.23
83	335.0	Heptadecanoic acid, met- (N,G)	1731-92-6	FA	0.14^{C}	1.17^{B}	0.17^{B}	1.019^{B}	0.19^{A}	1.07^{A}	-0.03	-0.38	0.71
75	289.1	Tridecanoic acid, 12-methyl- (N)	5129-58-8	FA	0.19^{C}	1.36^{A}	0.20^{BC}	1.198^{AB}	0.25^{A}	1.15^{A}	-0.04	0.12	0.71
77	309.9	3,7,11,15-Tetramethyl-2- (2) (N)	102608-53-7	O (AA)	0.45^{B}	1.23^{A}	0.49^{AB}	0.951^{AB}	0.49^{AB}	1.08^{AB}	-0.07	0.04	0.16

16	232.2	Malic acid, O-(trimethylsi- (N,G)	107241-82-7	OA	0.06^{B}	1.16^{A}	0.05^{C}	1.185^{C}	0.05^{C}	1.04^{C}	-0.14	0.85	-0.19
39	291.8	Ribonic acid, 2,3,4,5-tetr- (G,N)	57197-35-0	OA	0.14^{C}	1.34^{B}	0.15^{C}	1.349^{B}	0.28^{A}	1.11^{A}	-0.18	-0.20	0.88
88	347.9	Hexadecanoic acid, 2-[(t- (N,G)	21987-11-1	FA	0.09^{C}	1.40^{B}	0.11^{B}	1.267^{B}	0.18^{A}	1.08^{A}	-0.19	-0.26	0.83
90	352.7	Unidentified (327,97,75,111,83)	N/A	N/A	0.39^{C}	0.73^{D}	0.42^{C}	1.010^{C}	0.77^{A}	0.87^{B}	-0.29	-0.55	0.65
79	315.2	7,10,13-Hexadecatrienoic - (N)	56554-30-4	FA	1.63^{CD}	0.85^{D}	1.978^{AB}	0.730^{D}	2.24^{A}	0.84^{BC}	-0.35	-0.73	0.11
3	176.7	Acetic acid, [(trimethylsilyl)- (N)	33581-77-0	OA	0.08^{B}	1.19^{A}	0.05^{D}	1.137^{D}	0.04^{D}	1.53^{C}	-0.37	0.83	-0.07
91	358.8	Heptadecanoic acid, 15-me- (N)	57274-46-1	FA	0.04^{B}	0.84^{B}	0.04^{B}	0.874^{B}	0.05^{A}	0.70^{B}	-0.41	-0.36	0.12
80	318.7	9-Hexadecenoic acid, met- (N)	1120-25-8	FA	0.34^{B}	0.76^{C}	0.35^{B}	0.776^{C}	0.43^{A}	0.92^{AB}	-0.42	-0.69	0.21
78	314.3	7,10-Hexadecadienoic aci- (N)	16106-03-9	FA	0.17^{A}	0.83^{B}	0.17^{A}	0.754^{B}	0.20^{A}	0.88^{A}	-0.59	-0.50	0.14
41	294.1	Methylcitric acid, tetrak- (1)(N,G)	N/A	OA	0.39^{B}	0.98^{B}	0.22^{C}	1.074^{C}	0.42^{B}	1.18^{A}	-0.70	0.17	0.60
51	316.6	EIQTMS_N12C_LJAF_1912.2_22 36BN50_Galactose met- (G)	N/A	SR	15.07^{A}	0.75^{B}	7.17^{CD}	0.653^{D}	10.97^{B}	0.76^{BC}	-0.75	0.32	-0.20
50	313.7	D-Glucose, 2,3,4,5,6-penta- (N)	34152-44-8	SR	69.61^{A}	0.78^{B}	38.01^{B}	0.555^{C}	49.28^{B}	0.87^{B}	-0.80	0.30	-0.24
61	334.5	Unidentified (204,73,319,205,217)	N/A	N/A	0.45^{B}	0.94^{B}	0.23^{C}	0.698^{C}	0.60^{A}	0.63^{B}	-0.81	0.06	0.27
7	213.4	Silanamine, 1,1,1-trimeth- (N,G)	5630-81-9	O (P)	0.46^{A}	1.03^{A}	0.40^{B}	1.007^{B}	0.42^{AB}	1.10^{A}			
12	223.0	Butanedioic acid, bis(trim- (G,N)	40309-57-7	OA	1.03^{A}	0.99^{A}	0.55^{B}	1.033^{B}	0.38^{C}	1.16^{C}			
17	233.1	2(3H)-Furanone, dihydro-3- (N)	55220-79-6	N/A	0.12^{A}	1.07^{A}	0.077^{B}	1.144^{B}	0.06^{C}	0.91^{C}			

21	244.7	Unidentified (172,82,73,75,160)	N/A	N/A	0.05^{A}	1.14^{A}	0.06^{A}	1.144^{A}	0.05^{A}	1.05^{A}
27	262.9	á-Amino isobutyric acid tri-T- (N)	N/A	OA	0.09^{C}	1.16^{BC}	0.12^{AB}	1.034^{A}	0.12^{AB}	0.93^{AB}
33	277.2	2,4,5-Trihydroxypentanoic acid(4TMS) (G)	N/A	OA	0.90^{A}	1.00^{A}	0.89^{A}	0.943^{A}	0.94^{A}	1.02^{A}
34	278.2	Xylitol, 1,2,3,4,5-pentakis-O-(N)	14199-72-5	SR	1.02^{B}	1.17^{B}	1.56^{A}	1.105^{A}	1.20^{B}	1.02^{B}
35	279.2	EIQTMS_N12C_LJAF_1669.3_22 36BN50_Xylose metho- (G)	N/A	SR	0.23^{C}	1.03^{C}	0.40^{A}	1.029^{A}	0.29^{B}	1.04^{B}
37	286.9	EITTMS_N12C_SD1_1730.5_128 8EC39_Rhamnose met- (G)	N/A	SR	0.23^{B}	1.06^{B}	0.30^{A}	0.867^{AB}	0.26^{AB}	0.98^{AB}
38	290.0	D-Xylofuranose, 1,2,3,5-tet- (N)	56271-68-2	SR	2.37^{B}	1.04^{B}	2.37^{B}	0.978^{B}	2.90^{AB}	1.03^{A}
40	293.4	2-Keto-l-gluconic acid, p- (G,N)	N/A	OA	0.75^{A}	1.11^{A}	1.19^{A}	0.969^{A}	1.24^{A}	1.16^{A}
42	296.5	L-Gluconic acid, 2,3,5,6-tet- (N)	56298-43-2	OA	0.48^{C}	1.05^{BC}	0.52^{BC}	0.985^{BC}	0.59^{A}	0.94^{AB}
43	296.9	Methylcitric acid, tetra- (2)(G,N)	N/A	OA	3.92^{B}	0.98^{B}	3.93^{B}	1^{B}	4.89^{A}	1.09^{A}
44	298.9	Glucaric acid, 2,3,4,5-tetr- (G,N)	38165-96-7	OA	0.24^{B}	1.12^{B}	0.33^{A}	1.013^{A}	0.08^{C}	1.32^{C}
48	309.8	D-Fructose, 1,3,4,5,6-pe- (1) (N)	56196-14-6	SR	52.23^{BC}	0.92^{C}	36.37^{D}	0.948^{D}	61.01^{A}	0.93^{AB}
49	311.4	D-Fructose, 1,3,4,5,6-pe- (2) (N)	56196-14-6	SR	47.71^{A}	0.93^{A}	30.96^{B}	0.968^{B}	48.58^{A}	0.89^{A}
52	318.3	Trimethylsilyl ether of glu- (N,G)	14199-80-5	O (A)	0.45^{A}	1.17^{A}	0.50^{A}	1.142^{A}	0.52^{A}	1.12^{A}
53	320.4	à-D-Glucopyranoside, m- (G,N)	2641-79-4	SR	0.77^{A}	1.08^{A}	0.46^{B}	1.117^{B}	0.37^{B}	0.96^{B}

55	323.7	D-Turanose, heptakis(trim- (N)	60065-05-6	SR	0.44^{A}	0.98^{A}	0.13^{C}	1.345^{C}	0.35^{B}	0.91^{B}	
56	325.6	Galactonic acid, 2,3,4- (1)(N,G)	55400-16-3	OA	1.30^{A}	1.02^{A}	1.35^{A}	0.986^{A}	1.48^{A}	1.09^{A}	
57	328.6	Galactonic acid, 2,3,4- (2)(N,G)	55400-16-3	OA	0.55^{C}	0.85^{C}	1.10^{B}	0.885^{B}	1.88^{A}	1.08^{A}	
59	332.9	2-Propenoic acid, 2,3,3-tris- (N)	18741-99-6	OA	0.10^{B}	1.14^{B}	0.19^{A}	1.111^{A}	0.12^{B}	0.98^{B}	
64	345.2	Glucose oxime hexaTMS (N)	120850-89-7	SR	1.37^{B}	1.06^{B}	1.42^{B}	1.005^{B}	1.88^{A}	1.06^{A}	
65	348.5	Galactose oxime hexa- (1)(G,N)	120850-88-6	SR	0.23^{B}	1.07^{B}	0.19^{B}	1.084^{B}	0.38^{A}	1.01^{A}	
66	349.3	Galactose oxime hexa- (2)(G,N)	120850-88-6	SR	0.31^{BC}	1.08^{B}	0.25^{C}	1.001^{C}	0.54^{A}	0.10^{A}	
68	356.8	D-Glucose, 4-O-[2,3,4,6-tet- (N)	55669-93-7	SR	2.73^{BC}	1.10^{B}	2.46^{C}	1.161^{BC}	3.61^{A}	1.04^{A}	
69	379.0	Gulonolactone tetra-TMS, xs (N)	N/A	N/A	1.37^{B}	0.96^{B}	1.37^{B}	1.031^{B}	2.01^{A}	0.98^{A}	
70	382.0	D-Glucuronic acid, 2,3,4,5-t- (N)	55530-80-8	OA	0.64^{B}	1.11^{AB}	0.66^{B}	1.015^{B}	0.63^{B}	1.23^{AB}	
73	211.2	Benzene, 1,3-bis(1,1-dime- (N)	1014-60-4	O (AH)	0.07^{AB}	1.45^{A}	0.09^{B}	1.018^{AB}	0.11^{A}	0.80^{AB}	
81	319.5	Pentadecanoic acid, 14-m- (N)	5129-60-2	OA	5.10^{C}	1.09^{BC}	5.30^{C}	1.072^{BC}	6.13^{AB}	1.06^{A}	
82	330.5	Hexadecanoic acid, 14-me- (N)	2490-49-5	FA	0.04^{AB}	1.17^{A}	0.04^{AB}	1.026^{AB}	0.04^{B}	1.11^{AB}	
89	349.5	Octadecanoic acid, meth- (G,N)	112-61-8	FA	6.96^{B}	1.04^{B}	7.33^{B}	1.007^{B}	7.92^{A}	1.04^{A}	
93	377.0	Eicosanoic acid, methyl e- (N,G)	1120-28-1	FA	0.14^{B}	1.04^{AB}	0.22^{A}	1.006^{A}	0.16^{AB}	1.01^{AB}	
94	402.4	Dodecanoic acid, 10-methyl- (N)	5129-65-7	FA	0.03^{A}	0.99^{A}	0.02^{A}	1.171^{A}	0.03^{A}	1.03^{A}	
Percentage of Variance explained											37.99 20.25 16.16

1. Cultivars are L=Libertas; C=Caesar; R= Russet Burbank; P= pathogen inoculated; W= water inoculated; P/W= the ratio of abundances of pathogen over water-inoculated (P/W>1.0 is increased in abundance); different letters indicate significance within the row/metabolite among treatments at P=0.05 using Student-Newman-Keuls (SNK) test for means comparisons of the total abundances of the 6 treatments.

2 Metabolites: 1-72 found methanol-water extract; 73-94 found chloroform extract.

3 Retention Index (RI), calculated based on Eckel (2000).

4 Shortened names according to NIST (N = a letter in parenthesis at the end of the name), GOLM Metabolome Database (G) or any combinations (i.e. GN, NG), library with higher probability for the same metabolite is listed first.

5 CAS Registry Number = Chemical Abstract Service Registry Number.

6 GR= Chemical groups of compounds: AA= Amino Acid; FA= Fatty Acid; OA= Organic Acid; O (AH)= Other Aromatic Hydrocarbon; O (P)= Other Phenolic; Sugar=SR.

7 Factor-loadings of metabolites to F1, F2 and F3-scores, based on factor analysis of abundances of 106 significant metabolites; the loadings can be positive or negative.

Fig. 5.1. Scatter plot of treatments using factor scores based on factor analysis of the abundances of 60 metabolites. Cultivars: C = Caesar, L= Libertas, and R = Russet Burbank. Inoculations: W = Water-inoculated and P = Pathogen-inoculated. F1 = First Factor (Principal Component) that explained (38%) highly resistance function; F2 = Second Factor that explained (20%) moderately resistance function; F3 = Third Factor that explained (16%) susceptible function.

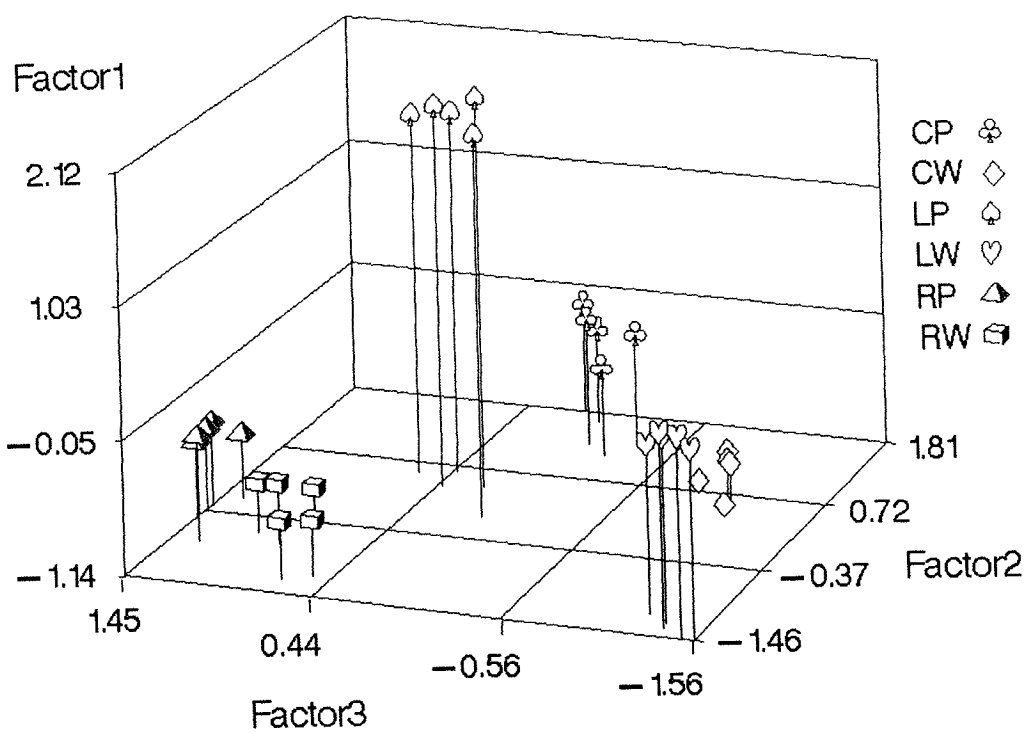
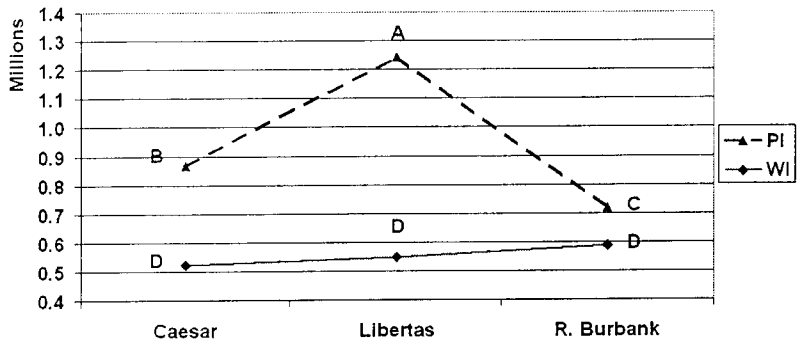
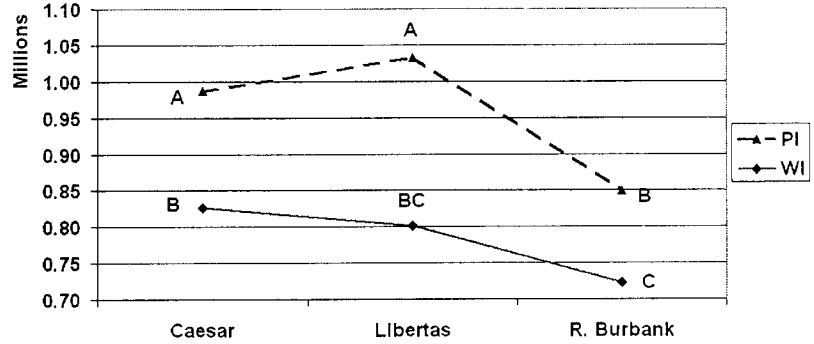

Factor1
2.12
1.03
−0.05
−1.14
1.45
0.44
−0.56
−1.56
Factor3
CP
CW
LP
LW
RP
RW
1.81
0.72
−0.37
−1.46
Factor2

Fig. 5.2. Examples of Defense Related metabolites (DR-metabolites); A, General Defense Related metabolites (GDR-metabolites); B, Resistance Specific Defense Related metabolites (RSDR-metabolites); C, Resistance Constitutive Defense Related metabolites (RCDR-metabolites); PI= Pathogen Inoculated, WI= Water Inoculated; Y-axis= Total abundance of the metabolites in millions; Means with the same letter in each metabolite are not significantly different at (P=.05) according Student-Newman-Keuls (SNK) test.

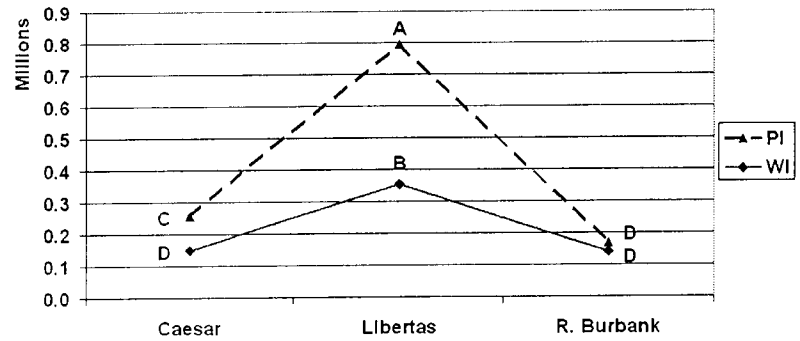
A1


L-Threonine (18)
Millions
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
A
B
C
D
D
D
PI
WI
Caesar
Libertas
R. Burbank

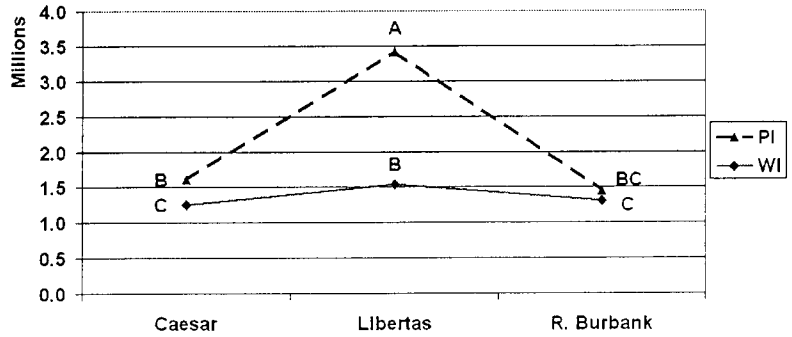
A2


Xylulose (29)
Millions
1.10
1.05
1.00
0.95
0.90
0.85
0.80
0.75
0.70
A
A
B
B
BC
C
PI
WI
Caesar
Libertas
R. Burbank

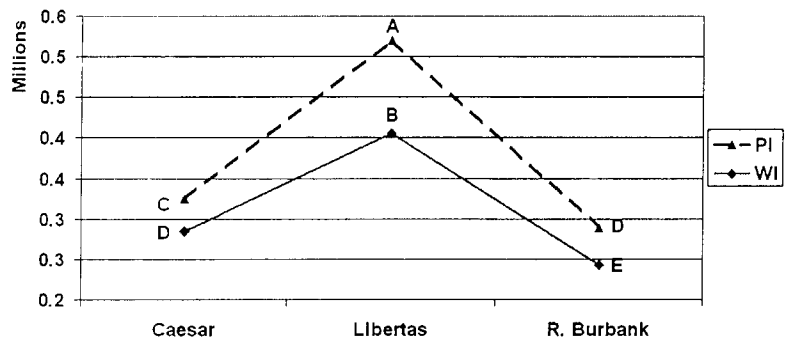
B1


L-Phenylalanine (32)
Millions
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
A
B
C
D
D
D
PI
WI
Caesar
Libertas
R. Burbank

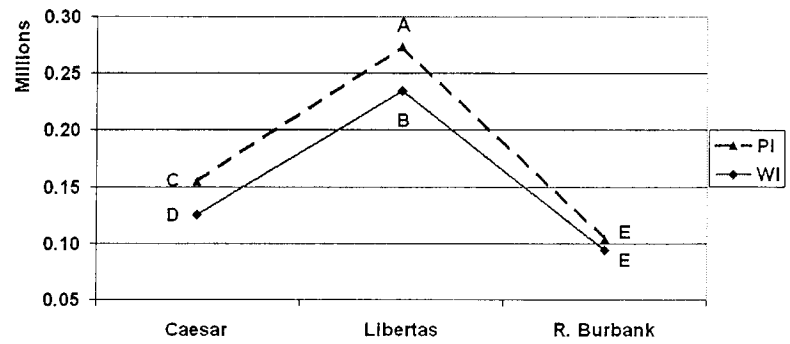
B2


Shikimic acid (45)
Millions
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
A
B
C
B
BC
C
PI
WI
Caesar
Libertas
R. Burbank

C1


Malonic acid (5)
Millions
0.6
0.5
0.5
0.4
0.4
0.3
0.3
0.2
A
B
C
D
D
E
Caesar
Libertas
R. Burbank
PI
WI

C2


Fumaric acid (14)
Millions
0.30
0.25
0.20
0.15
0.10
0.05
A
B
C
D
E
E
Caesar
Libertas
R. Burbank
PI
WI

5.5. Discussion

The present study reports the potential use of metabolic profiling as a tool to phenotype potato cultivars varying in horizontal resistance to *P. infestans*. The abundances of metabolites detected here were subjected to ANOVA to select the PR-metabolites, which in turn were subjected to factor analysis, using principal components, to identify classes. These classes were related to resistance of cultivars based on disease severity to identify the hidden host-pathogen interaction functions. The biological functions were further explained based on the metabolites that loaded high to the F-vector that identified the function.

The three potato cultivars used here varied significantly in horizontal resistance based on disease severity. The factor analysis of metabolic profiles of these cultivars classified the cultivars into distinct clusters or metabolic phenotypes similar to those based on disease severity phenotypes, meaning the resistance in potato to *P. infestans* can be metabolically phenotyped. Of course, more cultivars varying in resistance have to be evaluated to confirm this concept. Factor analysis of 60 PR-metabolites identified three functions. The high resistance function (F1-vector), that discriminated high resistance in cultivar Libertas, was associated with 30 DR-metabolites. Among these 9 were common to all the three cultivars, meaning they share common defense mechanism(s). The moderately resistance function (F2-vector) discriminated moderately resistant cv. Caesar from the rest, the highly resistant Libertas and susceptible Russet Burbank. Ten metabolites had high loading to F2-vector. The susceptible function (F3-vector) discriminated Russet Burbank from the rest. Fourteen metabolites had high loading to F3-vector.

FACTOR analysis results showed that each cultivar developed unique defense mechanism(s) and distinctive strategy/strategies upon infection by *P. infestans* and each cultivar could be independently explained as it was spatially separated by one of the

orthogonal factors. In addition, the tested cultivars were found to share some common strategies and activate some common pathways in response to this pathogen. Referring to the ANOVA analysis, Libertas, the most resistance cultivar, developed the smallest lesions after the infection by *P. infestans* (Table 5.1). Therefore, It became apparent that Libertas evolved the most efficient means or tactics to defer this pathogen. Thus, our discussion will be concentrated on the metabolites produced by Libertas and the levels of up-regulation of these metabolites in the other two cultivars will be explored

In response to pathogens, plants develop different strategies to recognize and to defer pathogens involving mechanical and biochemical barriers (Montesano, 2003; Osbourn, 1996a,b). In potato tubers, after the pathogen attack, the primary and secondary metabolisms have noticeably changed and the accumulation of defense related metabolites including oxidative burst, compounds from the phenylpropanoid pathway and phytoalexins have been reported (Nakane et al., 2003). In potatoes, although different levels of secondary metabolites could be produced by different cultivars, we believe that changes in the primary metabolism of the plant could provide a solid foundation for discriminating different levels of resistance in different cultivars/breeding lines.

The resistance function metabolites, with high loading to the first factor vector, identified here can be used as resistance defense biomarker metabolites, even though confirmation based on significance among cultivars and validation using other resistant cultivars are required, to exploit these for screening breeding lines for resistance. Several of the detected metabolites here were highly activated following the pathogen inoculation and many of them are precursors for the production of several antimicrobial compounds, signal molecules or precursors that produce these metabolites. The OAs, fumaric (Fig.

5.2 C2), malic and isocitric acids, detected here, had the highest abundances in the pathogen-inoculated Libertas. These metabolites are intermediates of the Krebs Cycle that is directly involved in the production of different AAs belonging to Glutamic and Aspartic acid families. Consequently, the abundances of the AAs, L-Isoleucine, L-threonine (Fig. 5.2 A1), and L-Aspartic acid that belong to the Aspartic acid family and the AAs Proline and Glutamine of the Glutamic acid family were highly increased following pathogen inoculation in Libertas compared to Caesar and Russet Burbank. These AAs are the primary building blocks in the production of different PR-proteins and enzymes. L-proline is involved in the production of extensin, a sub-group of hydroxyproline-rich glycoproteins (HRGPs) family, that has the ability to increase the rigidity of the plant cell walls by cross-linking different cell wall components such as pectin (Jackson et al., 2001). In water-stressed tomato leaves, an increase in glutamine, glutamate and asparagine AAs have been reported (Bauer et al., 1997). Similarly, high levels of the AAs of the glutamate and aspartate families were found in invitro-grown potato tubers compared to soil-grown tubers (Roessner et al. 2000). The abundance of Shikimic acid (Fig. 5.2 B2) was highly increased after the pathogen inoculation in the resistant cultivar Libertas, followed by Caesar then Russet Burbank (Table 5.2). High levels of AAs (i.e. valine, leucine, threonine), OAs (i.e. fumaric acid and shikimic acids) were reported to be elevated in concentration after the elicitation of cell cultures of *Medicago truncatula* by methyl jasmonate and yeast elicitor (YE) (Broeckling et al., 2005). Shikimic acid is the precursor of the aromatic AAs Phenylalanine (Fig. 5.2 B1) and Tyrosine. These two AAs were highly increased in abundances in Libertas, and are the precursors of the Phenylpropanoid Pathway that produces many plant secondary defense metabolites including: phenolics, lignins and hydrolyzable tannins (Dixon et al.,

2002; Nakane et al., 2003; Barabasi and Oltvai, 2004). Some products of the phenylpropanoid pathway along with Malonic acid combined to produces several flavonoids (i.e. coumarines, flavones, isoflavones, isoflavanones) and condensed tannins (Croteau et al., 2000). Many of these secondary metabolites are important in deterring plant pathogens (http://www.scri.sari.ac.uk/TiPP/pps/Chart.pdf). Malonic acid (Fig. 5.2 c1) and its derivatives Methyl malonate [2] were also highly increased in abundances after the pathogen inoculation in Libertas, followed by Caesar and Russet Burbank, respectively. Moreover, Malonic acid is a precursor of the FA Octadecanoic acid (Stearic acid) that was also highly increased in abundance in Libertas.

Referring to the phenolic compounds, Benzoic acid [74], a precursor of the plant-signal metabolite Salicylic acid, that provokes many defense responses of the infected plants (Hammond-Kosack and Jones, 1996), was also found to have the highest up-regulation ratio in Libertas followed by Caesar and Russet Burbank, respectively. Also, Silanamine [62] and Norepinephrine [67] were found to have the highest abundances in the pathogen-inoculated Libertas. The Sugar metabolite, Xylulose [29; Fig. 5.2 A2] and the OA, D-Gluconoic acid [58] were found to have the highest up-regulation levels in Libertas. These metabolites are precursors of the Pentose Phosphate Pathway (PPP) that produces erythrose-4-phosphate, an important precursor for the production of the aromatic amino acids. An increase rate of respiration, accumulation of CO2, and the activation of the PPP were reported in parsley plant after the inoculation by *Phytophthora megasperma* (Norman et al., 1994). In summary, the shikimic acid pathway, Pentose Phosphate Pathway, Citric Acid Cycle and amino acid production pathways were highly activated in Libertas, the most resistant cultivar. Such a scale-free activation has been proposed by Barabasi and Oltvai (2004).

Among the 30 DR-metabolites loading to F1-vector nine metabolites were common and significantly increased in abundances after the pathogen inoculation in the three tested cultivars and the highest abundances were reported in the resistance cultivar, Libertas. This indicates that the tested cultivars, with different levels of the horizontal resistance, share and activate some common metabolites/pathways in response to the pathogen attack. The activation of these pathways was found to be higher in Libertas, the most resistant cultivar. On the other hand, there were more shared common pathways, as described earlier, between the Moderately resistant cultivars and the highly resistance cultivars. 26 metabolites (RSDR-metabolites) were found to be in common between Libertas and Caesar but the up-regulation levels after the pathogen-inoculation was always higher in Libertas. This is an interesting point indicating that the highly resistant cultivar, Libertas, is more efficient in up-regulating certain metabolites and defense pathways than the less resistant cultivars. Metabolic profiling, a large-scale metabolite quantification, is an evolving field of systems biology and there are several limitations. There is no single extraction method to extract thousands of metabolites produced by plants (Bino et al., 2004). The great diversity in the chemical structures and the concentration of metabolites in a tissue is highly variable, and there is no single platform to analyze all metabolites in one run. However, GC-MS is still considered to be the one of the cheapest and best platforms available (Sumner et al., 2003; Dunn et al., 2005). In spite of such limitations we were able to discriminate resistance in potato to late blight pathogen based on metabolic profiling using GC/MS. On the other hand, it is possible that several metabolites that can better explain the resistance functions were not explored in this study.

In conclusion, we were able to discriminate three potato cultivars varying in levels of horizontal resistance based on metabolic profiling. Though the metabolites detected here didn't vary qualitatively among different cultivars, they varied in their abundances. Several DR-metabolites were significantly increased in abundances following pathogen inoculation in the highly resistant cultivar Libertas than in other less resistant cultivars, Caesar and Russet Burbank. The cv. Libertas highly activated several OAs, AAs and phenolic compounds. The high levels of some of these DR-metabolites, especially, shikimic acid, tyrosine and phenylalanine are direct indicators for the activation of the Phenylpropanoid Pathway. Malonic acid pathway was also predicted to be activated since high levels of Malonic acid was detected. These two pathways are known for their role in the production of a wide range of secondary metabolites in response to stress and pathogen attack. The moderately resistance cultivar, Caesar, was found to express most of the DR-metabolites that increased in abundances in liberates but with relatively lower abundances. On the contrary, fewer metabolites and with low abundances have been activated in the susceptible cultivar Russet Burbank. The 30 DR-metabolites with the highest abundances in Libertas can be considered as resistance biomarker metabolites and further exploited for use in screening resistance against late blight, following further confirmation using more cultivars. Thus, there is potential to use metabolic profiling as a tool for high throughput screening of potato cultivars to discriminate horizontal resistance against late blight, and can be used as an additional or alternative method to that based on disease severity phenotypes.

5.6. Acknowledgements

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CHAPTER 6

GENERAL DISCUSSION, CONCLUSIONS, AND SUGGESTIONS FOR FUTURE RESEARCH

Metabolic profiling is an evolving tool for studying biology of plants and other living organisms. Metabolites are the end products of genes (Fiehn, 2002) and metabolic studies should be explored for better understanding of functional genomics (Sumner et al., 2003). Metabolic profiling has been used by many researchers to study genetically modified crops (Roessner et al 2000, 20001a,b; Fiehn et al., 2000b;), biotic and a biotic-stressed plants (Johnson et al., 2003; Broeckling et al., 2005) and to discriminate wheat cultivars varying in the quantitative resistance to fusarium head blight (Hamzehzarghani et al., 2005).

The studies conducted here report metabolic profiling as a tool to discriminate horizontal resistance in potato against late blight. For metabolic profiling of potato plant, the metabolite extraction protocols developed by Fiehn et al., (2000) were adopted in the 4th and the 5th chapters. In the 4th chapter, the potato cv. AC Novachip was inoculated with *P. infestans* and samples were collected 24, 48 and 96 hours after water or pathogen inoculation. The main objective of the 5th chapter was to study the temporal dynamics of the metabolites after the pathogen inoculation and to identify the plausible pathways of their productions. Results showed elevation of the abundances of many metabolites over time, especially, amino, fatty and organic acid between the water inoculated and the pathogen-inoculated plants. Immediately after the pathogen germination the penetration peg starts piercing through the plant cuticle, lots of morphological changes in the plant cells were reported including the movement of the host nucleus to the site of the

pathogen penetration, streaming of the cell cytoplasm, changes in the actin cytoskeleton, and the deposition of papillae between the cell wall and the cell membrane. This papilla contains callose and assist in the cell wall reinforcement (Schmelzer, 2002). In the compatible interactions and during the biotrophic phase, the pathogen secretes less toxic compounds in order not to evoke the defense responses of the host (Mendgen and Hahn, 2002). After the inoculation of *P. infestans* to susceptible, resistant and non-host plants, all plants responded in a similar way and activated the hypersensitive response (HR), but the speed of this response varied among the plants. In the non-host and the completely resistant *Solanum* spp., the response was rapid and the pathogen killed very fast in less than 24 hours, and the death of 1-3 cells around the pathogen was seen. In the partially resistant cultivars, the response was slower and extended 16-46 h, and 5 or more cells were killed around the pathogen hypha. In the susceptible cultivars, the pathogen hyphae were able to escape death and were able to establish a biotrophic invasion in the mesophyll cells. After 46 hours the pathogen became more aggressive as it enters the necrotrophic phase and the sporangiophores of the pathogen were seen emerging through the plant stomata (Vleeshouwers et al., 2000). In response to this progress, plants evolved sophisticated and dynamic defense responses to defer the pathogen and that what we have proven in our studies at the biochemical levels. In the first day after the pathogen inoculation (Chapter 4, Factor 2), the activation of certain amino acids valine, and isoleucine belonging to the aspartate, and alanine families were found. These amino acids are precursors for the production of antimicrobial compounds (Coruzzi and Last, 2000). Also, the abundance of proline was increased in the first and second day after the pathogen inoculation. Proline is involved in the production of proline-rich proteins (PRPs, Showalter, 1993), hydroxyproline-rich glycoproteins (HRGPs), and extensins

(Jackson et al., 2001). On the other hand, a drop in the total abundances of some sugars was noticed. Part of these metabolites could have been produced to strengthen the cell walls of the infected cells and to prevent the pathogen penetration as they are associated with the production of callose and papillae (Vleeshouwers et al. 2000). In the second day after inoculation, a shift in the produced metabolites was noticed (4^{th} chapter F3 metabolites) and that includes the activation of proline, glutamine and mainly the aromatic amino acids phenylalanine and tyrosine. These metabolites are precursors of the phenylpropanoid pathway that is known to be highly activated in response to pathogen attack (Dixon et al., 2002). In the 4^{th} day after inoculation, the collapse of the primary and secondary defense responses was noticed and was accompanied by the transition of the pathogen from the biotrophic to necrotrophic phase. In chapter 5, the metabolites were analyzed after 48 hours of the pathogen attack in 3 cultivars having different levels of horizontal resistance. The main objective was to discriminate these cultivars according to their metabolic profiles and to find marker metabolites associated with the most resistant cultivar, Libertas. The tested cultivars were found to activate different pathways and to evoke different defense responses to postpone the pathogen attack. In this study only the PR-metabolites were used in the FACTOR analysis and the main objective was to find the correlation between metabolites that were increased or decreased in abundances after the pathogen inoculation and to study their biological functions in different cultivars. 60 PR-metabolites were analyzed and three main factors were able to explain 75% of the total variance and to discriminate the 3 cultivars. F1 was the most important factor and explained 38% of the total variation. This factor were highly associated with the pathogen inoculated Libertas, the most resistant cultivar. F2 and F3 explained the moderately and the susceptible functions, respectively. The three factors

are orthogonal and they are totally independent. In other words, each group of metabolites loaded to these factors was independent also and should have been activated/produced by different pathways. In the most resistant cultivar, 30 DR-metabolites were reported and 9 of these metabolites were increased in abundances in the three cultivars after the pathogen inoculation (GDR-metabolites) and 26 metabolites were increased in abundances after the pathogen inoculation of the moderately and the resistant cultivars (RSDR-metabolites). Because these metabolites had highly loadings for the same factor and highly correlated, this is an indication that they had common pathways of productions. Even though these metabolites were common between the different cultivars, the highest abundance was always recorded in the resistant cultivar, Libertas. This illustrates the ability of the resistant cultivars to produce high amounts of such precursors that are involved in the defense responses. The DR-metabolites were found to be precursors for the shikimic acid, Pentose Phosphate, and malonic acid pathways that produce several secondary defense metabolites (Croteau et al., 2000; Dixon et al., 2002; Nakane et al., 2003). On the other hand, the DR-metabolites and the 13 RCDR-metabolites reported in Libertas can be considered as biomarkers for the high resistance levels and could be targeted in the pre-selecting breeding programs.

Recommendations for further studies

1. For a comprehensive metabolic profiling of most plant metabolites, different solvents and extraction procedures must be used.
2. Because the metabolites are found in different concentrations in the plant tissues (Dunn et al., 2005), and each instrument has a specific dynamic range (reviewed by Sumner et al., 2003), more than one instrument should to be used to increase

the number of the extracted metabolites. For example Broeckling et al., (2005) used GC/MS to study the plant primary metabolism and the LC-MS to study the secondary metabolism.

3. In this thesis, different DR- and RCDR-metabolites were reported in the resistant cultivar Libertas. To have a comprehensive list of DR-metabolites, many cultivars with high levels of resistant must be evaluated. On the other hand, the cultivars must be tested at different intervals after the pathogen inoculation because the speed of producing the DR-metabolites is crucial during the dense responses.
4. In searching for durable horizontal resistance, the use of cultivars with known resistance QTLs is very helpful. For example, the strongest QTLs for resistance against *P. infestans* were reported to be located on chromosomes XII, V, III, VIII, X, IV, and II, respectively (Sliwka, 2004). It would be very useful to find the metabolites associated with each of these QTLs. Careful should be taken her as some of the R-genes (major genes associated with the vertical resistance) were reported to be essential for the full expression of certain QTLs.
5. The use of the functional genomic approach, transcriptomics, proteomics and the plant metabolomics is necessary to better understanding of the function of the genes (Fiehn, 2001; Sumner et al., 2033; Nakane et al., 2003; German et al., 2005). Kolbe et al., (2006) used the metabolic profiling approach in combination of transcriptome to study the metabolic change in the leaves of *Arabidopsis thaliana* in response to altering the thiol-disulfide status by feeding the plant by low concentrations of dithiothreitol (DTT), a non-physiological substrate for thioredoxin. Results showed a dynamic change in the plant transcriptome and

metabolome including the increase in the concentrations of certain amino acids, shikimic acid, starch and the reduction in glucose flux.

6. It is important to use new column in GC when analyzing plant samples. High bleeding in old columns increases the background noises and masks metabolites in many cases. This was clearly seen in the third study at high oven temperatures. Some metabolites such as á-Sitosterol, Stigmasterol, and Solanidine that are separated at high temperatures and eluted late were difficult to be quantified in the third study while they were clearly detected in the second study. Fiehn et al., (2000) reported that after 150 runs the bleeding in DB-5 columns was very high and he suggested the use of SPB50 column that could be used to run up to 1000 samples.

CHAPTER 7

CONTRIBUTIONS TO KNOWLEDGE

With the accumulation of knowledge in the area of genomics there is a great demand for knowledge in the area of functional genomics. Though transcriptome and proteome data can provide essential knowledge on pathways of biological action, for a comprehensive understanding of the gene expression and the effect on pathogen suppression data on metabolites are very important (Fiehn et al. 2001; Sumner et al., 2003). Furthermore, since the metabolites are often the end points of metabolism that usually provide plant defense compounds we have used metabolic profiling to phenotype cultivars varying in horizontal resistance. Accordingly, our studies were focused to metabolic phenotype potato cultivars varying in horizontal resistance to late blight. Though both leaves and tubers are susceptible for infection we have chosen leaves as most fungicides are applied to manage leaf blight. Cultivars varying in horizontal resistance were inoculated with water or pathogen and metabolites were profiled. Some of the specific contributions are:

1. Reports tentative identification of more than 100 metabolites in potato leaf-late blight interaction.
2. Identification of several PR-metabolites based on ANOVA and use of FACTOR analysis to identify sets of metabolites that explained pathogenesis function in a potato cultivar susceptible to late blight (chapter 4).

3. Provided proof on changes in metabolic pathways that lead to the activation of several metabolites in higher rates following pathogen inoculation of the resistant and the moderately resistant cultivars compared to water inoculation.
4. Proof that changes in the plant primary metabolism after the pathogen inoculation could be used to discriminate different levels of the horizontal resistance (chapter, 5).
5. Identification of several PR-metabolites based on ANOVA, and DR-metabolites, GDR-metabolites, RSDR-metabolites, and RCDR-metabolites that explained the resistance functions of potato-late blight interaction based on factor analysis.
6. The DR- and RCDR-metabolites identified here have the potential to be used as resistance related biomarkers for screening breeding lines for resistance against late blight. The sets of metabolites associated with resistance functions was involved collectively to produce antimicrobial compounds that have been previously reported to be involved in plant defense.
7. Provided proof on how potato cultivars with high level of horizontal resistance differ from those with lower levels, including susceptibility at the biochemical level.
8. Reports a novel technology 'Metabolic phenotyping of resistance in potato against late blight based on GC/MS' with potential for high through put screening of breeding lines for resistance against late blight, which also explained the mechanism of resistance.
9. Reports minor modifications of extraction of both polar and non-polar metabolites developed by Fiehn et al (2000a,b).

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Appendix 1. List of metabolites, Synonyms and boiling points for the most important metabolites reported in this thesis.

CAS NO.[1]	Metabolite Name[2]	Other Common Names	Boiling Points[3]
1014-60-4	Benzene, 1,3-bis(1,1-dimethylethyl)- (N,G)	1. Benzene, m-di-tert-butyl-. 2. m-Di-tert-butylbenzene. 3. 1,3-Di-tert-butylbenzene. 4. 1,3-Ditertiarybutylbenzene.	223.6±10.0
1120-25-8	9-Hexadecenoic acid, methyl ester, (Z)- (N)	1. Methyl palmitoleate. 2. Methyl palmitoleinate. 3. Palmitoleic acid, methyl ester.	394.2±0.0
1120-28-1	Eicosanoic acid, methyl ester (N,G)	1. Methyl arachate. 2. Methyl eicosanoate. 3. Arachidic acid methyl ester.	375.0±5.0
112-39-0	Hexadecanoic acid, methyl ester (N)	1. Palmitic acid, methyl ester. 2. n-Hexadecanoic acid methyl ester. 3. Methyl hexadecanoate. 4. Methyl palmitate. 5. Methyl n-hexadecanoate.	332.1±0.0
112-61-8	Octadecanoic acid, methyl ester (G,N)	1. Stearic acid, methyl ester. 2. n-Octadecanoic acid, methyl ester. 3. Methyl ester of octadecanoic acid.	355.5±0.0

		4. Methyl n-octadecanoate. 5. Methyl stearate.	
112-62-9	9-Octadecenoic acid (Z)-, methyl ester (N)	1.Oleic acid, methyl ester. 2. Emery, oleic acid ester. 3. Oleic acid, methyl ester, cis 4. Methyl oleate. 5.Methyl (Z)-9-octadecenoate	351.4±0.0
112-63-0	9,12-Octadecadienoic acid (Z,Z)-, methyl ester (N,G)	1. Linoleic acid, methyl ester. 2. Methyl cis,cis-9,12-octadecadienoate. 3. Methyl linoleate. 4. Methyl octadecadienoate. 5. Methyl 9-cis,12-cis-octadecadienoate.	373.3±21.0
1188-74-5	Hexadecanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester (N)	1. Palmitic acid, 2,3-bis(trimethylsiloxy)propyl ester. 2. 1-Monopalmitin trimethylsilyl ether. 3. 1-Monopalmitoylglycerol trimethylsilyl ether.	471.9±35.0
1188-75-6	Octadecanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester (N)	1.Stearic acid, 2,3-bis(trimethylsiloxy)propyl ester	497.3±35.0
14330-97-3	1,2,3-Propanetricarboxylic acid, 2-[(trimethylsilyl)oxy]-, tris(trimethylsilyl)	1.Isocitric acid (tms)	401.1±45.0

	ester (N)		
16106-03-9	7,10-Hexadecadienoic acid, methyl ester (N)	Methyl 7,10-hexadecadienoate	346.1±21.0
15985-07-6	Glutamine, tris(trimethylsilyl)- (G,N)	1. L-Glutamic acid, N-(trimethylsilyl)-, bis(trimethylsilyl) ester . 2. Glutamic acid, N-(trimethylsilyl)-, bis(trimethylsilyl) ester 3. Glutamic acid, N-(trimethylsilyl)-, bis(trimethylsilyl) ester, L- .	326.9±37.0
1731-92-6	Heptadecanoic acid, methyl ester (N,G)	1. Margaric acid methyl ester. 2. Methyl heptadecanoate. 3. Methyl margarate. 4. n-Heptadecanoic acid methyl ester.	337.1±5.0
17596-96-2	Propanoic acid, 2-[(trimethylsilyl)oxy]-, trimethylsilyl ester (N,G)	1. Propionic acid, 2-(trimethylsiloxy)-, trimethylsilyl ester. 2. Bis(trimethylsilyl)lactate. 3. Lactic acid, bis(trimethylsilyl)oxy-, ester.	193.5±23.0
17962-03-7	2-Butenedioic acid (E)-, bis(trimethylsilyl) ester (G,N)	1. Fumaric acid, bis(trimethylsilyl) ester. 2. Bis(trimethylsilyl) fumarate. 3. Fumaric acid (tms)	224.0±23.0

18457-04-0	Propanedioic acid, bis(trimethylsilyl) (N,G)	1.Malonic acid, bis(trimethylsilyl) ester 2.Malonic acid (tms) 3.Bis(trimethylsilyl) malonate	232.6±0.0
18748-91-9	Octadecanoic acid, trimethylsilyl ester (N,G)	1. Stearic acid, trimethylsilyl ester. 2. Trimethylsilyl ester of Octadecanoic acid.	394.1±11.0
2490-49-5	Hexadecanoic acid, 14-methyl-, methyl ester (N)	1. Palmitic acid, n-methyl-, methyl ester. 2. Methyl 14-methylhexadecanoate	326.5±10.0
2582-79-8	Myo-Inositol, 1,2,3,4,5,6-hexakis-O-(trimethylsilyl)- (G,N)	1.Inositol, 1,2,3,4,5,6-hexakis-O-(trimethylsilyl)-, myo-	466.3±45.0
27844-07-1	l-Alanine, N-(trimethylsilyl (N,G)	1.Alanine, N-(trimethylsilyl)-, trimethylsilyl ester, l-	201.1±23.0
301-00-8	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)- (G,N)	1. Linolenic acid, methyl ester. 2. Methyl all-cis-9,12,15-octadecatrienoate. 3. Methyl linolenate.	364.4±0.0
30274-77-2	L-Proline, 5-oxo-1-(trimethylsilyl)-, trimethylsilyl ester (N,G)	1.Proline, 5-oxo-1-(trimethylsilyl)-, trimethylsilyl ester. 2.Pyroglutamic acid, bis(trimethylsilyl)-. 3.Pyroglutamic acid, di(trimethylsilyl)-	285.2±29.0
33581-77-0	Acetic acid, [(trimethylsilyl)oxy]-, trimethylsilyl ester (N)	1. Acetic acid, (trimethylsiloxy)-, trimethylsilyl ester. 2. Trimethylsilyl (trimethylsilyloxy)acetate.	176.5±23.0

		3. Trimethylsilyl trimethylsiloxyacetate.	
38191-87-6	Propanoic acid, 2,3-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester (N,G)	Propanoic acid, 2,3-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester (S.F).	254.8±30.0
38191-88-7	2,3,4-Trihydroxybutyric acid tetraTMS (N)	Butanoic acid, 2,3,4-tris[(trimethylsilyl)oxy]-, trimethylsilyl ester, (R*,R*)- (9CI)	334.0±42.0
40309-57-7	Butanedioic acid, bis(trimethylsilyl) (G,N)	1. Succinic acid (tms) 2. Bis(trimethylsilyl) succinate	237.7±23.0
40309-57-7	Butanedioic acid, bis(trimethylsilyl) ester (G,N)	1. Succinic acid (tms). 2. Bis(trimethylsilyl) succinate, (S.F).	237.7±23.0
51220-73-6	L-Tyrosine, N,O-bis(trimethylsilyl)-, trimethylsilyl ester (N,G)	N/A	378.8±42.0
55191-53-2	(R*,S*)-3,4-Dihydroxybutanoic acid triTMS (N)	1.Butanoic acid, 3,4-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester	282.4±30.0
55268-53-6	L-Aspartic acid, N-(trimethylsilyl)-, bis(trimethylsilyl) ester (G,N)	1. Aspartic acid, N-(trimethylsilyl)-, bis(trimethylsilyl) ester	305.4±37.0
55520-78-0	1-Cyclohexene-1-carboxylic a (N)	1. Shikimic acid (4TMS) (G) 2. 1-Cyclohexene-1-carboxylic acid, 3,4,5 (N,)	392.5±42.0
55520-89-3	Hexadecanoic acid, trimethylsilyl ester (N,G)	1. Palmitic acid, trimethylsilyl ester	364.3±11.0
55530-71-7	1-Propene-1,2,3-tricarboxylic acid,	1.cis-Aconitic acid (tms)	344.7±42.0

	tris(trimethylsilyl) ester, (Z)- (N)		
55556-99-5	Silanamine, 1,1,1-trimethyl-N-(trimethylsilyl)-N-[2-[(trimethylsilyl)oxy]-2-[4-[(trimethylsilyl)oxy]phenyl]ethyl]- (G,N)	1.Tetrakis-trimethylsilyl derivative of octopamine	408.7±55.0
5630-82-0	Glycine, N,N-bis(trimethylsilyl)-, trimethylsilyl ester (G,N)	1. Silanol, trimethyl-, ester with N,N-bis(trimethylsilyl)glycine (S.F)	259.4±42.0
56554-30-4	7,10,13-Hexadecatrienoic acid, methyl ester (N)	N/A	344.8±21.0
57-10-3	Hexadecanoic acid (N)	1. Palmitic acid. 2. n-Hexadecoic acid. 3. Coconut oil fatty acids. 4. Pentadecanecarboxylic acid, 5. Cetylic acid.	340.6±5.0
73090-79-6	Cystathionine-diTMS (N)	1.L-Homocysteine, S-[2-amino-3-oxo-3-[(trimethylsilyl)oxy]propyl]-, trimethylsilyl ester, (R)- (S.F)	380.3±42
7364-44-5	L-Valine, N-(trimethylsilyl)-, trimethylsilyl ester (N,G)	1. Valine, N-(trimethylsilyl)-, trimethylsilyl ester, L-. 2. N-(Trimethylsilyl)-L-valine trimethylsilyl ester	236.4±23.0

7364-47-8	L-Proline, 1-(trimethylsilyl)-, trimethylsilyl ester (N,G)	1.Proline, 1-(trimethylsilyl)-, trimethylsilyl ester, L-	263.7±29.0
7364-48-9	L-Serine, N,O-bis(trimethylsilyl)-, trimethylsilyl ester (G,N)	1. Alanine, 3-(trimethylsiloxy)-N-(trimethylsilyl)-, trimethylsilyl ester, L-. 2. N,O,O-Tris(trimethylsilyl)-L-serine. 3. Tris(trimethylsilyl)-L-serine.	277.2±30.0
7364-51-4	N,O-Bis(trimethylsilyl)-L-phenylalanine (G,N)	1. L-Phenylalanine, N-(trimethylsilyl)-, trimethylsilyl ester.. 2. Bis(trimethylsilyl)phenylalanine. 3. Alanine, 3-phenyl-N-(trimethylsilyl)-, trimethylsilyl ester, L- 4. N-(Trimethylsilyl)-L-phenylalanine trimethylsilyl ester.	324.4±42.0
7483-92-3	L-Isoleucine, N-(trimethylsilyl)-, trimethylsilyl ester (N,G)	1.Isoleucine, N-(trimethylsilyl)-, trimethylsilyl ester, L-	255.0±23.0
7537-02-2	N,O,O-Tris(trimethylsilyl)-L-threonine (G,N)	1. L-Threonine, N,O-bis(trimethylsilyl)-, trimethylsilyl ester. 2. Butyric acid,3-(trimethylsiloxy)-2-[(trimethylsilyl)amino]-, trimethylsilyl ester.	290.9±30.0

80-78-4	Solanid-5-en-3-ol, (3á)- (N)	1. Solanidine. 2. Solanidin. 3. Solatubine. 4. Solatubin. 5. 3-á-Solanid-5-en-3-ol. 6. 22R,25S-Solanidanine.	503.1±35.0
915-05-9	á-Sitosterol acetate (N)	1. Sitosteryl acetate. 2. á-Sitosterol 3-acetate. 3. Stigmast-5-en-3á-yl acetate. 4. Acetyl-á-sitosterol. 5. Sitosterol,á, acetate. 6. Stigmast-5-en-3á-ol, acetate.	514.5±29.0
929-77-1	Docosanoic acid, methyl ester (N)	1. Behenic acid, methyl ester. 2. Methyl behenate. 3. Methyl docosanoate. 4. n-Docosanoic acid methyl ester.	398.0±5.0

[1] CAS NO. = Chemical Abstract Service Registry Number.

[2] Shortened names according to NIST (N = a letter in parenthesis at the end of the name), GOLM Metabolome Database (G) or any combinations (i.e. GN, NG), library with higher probability for the same metabolite is listed first.

[3] Boiling Points in degrees Celsius

Appendix 2. List of abbreviations

2D-PAGE	Two-Dimensional Polyacrylamide Gel Electrophoresis
4CL	4-Coumarate CoA Ligase
AAFC	Agriculture And Agri-Food Canada
AAs	Amino Acids
ADP	Adenosine Diphosphate
AFLP	Fragment Length Polymorphism
AMP	Adenosine Monophosphate
AMV	Alfalfa Mosaic Virus
AP	AC Novachip Pathogen Inoculated
AUDPC	Area Under the Disease Progress Curve
avr	Avirulent
AW	AC Novachip Water Inoculated
BA	Benzoic Acid
BTH	Benzothiadiazole
cAMP	Cyclic Adenosine Monophosphate
CE	Capillary Electrophoresis
CE/MS	Capillary Electrophoresis/ Mass Spectrometry
CE/UV	Capillary Electrophoresis/Mass Spectrometry
CFIA	Canadian Food Inspection Agency
CoA	Coenzyme A
CP	Caesar Pathogen-Inoculated
CW	Caesar Water Inoculated

DAG	Diacylglycerol
DAHP	3-Deoxy—D-Arabino-Heptulosonate-7-Phosphate
DAI	Day After Inoculation
DINA	Dichloroisonicotinic Acid
DMAPP	Dimethyallyl Diphosphate
DNA	Deoxyribonucleic Acid
cDNA	Complementary Deoxyribonucleic Acid
dnOPDA	Dinor-Oxo-Phytodienoic Acid
DR	Defense Related
EC	Enzyme Commission Number
ER	Endoplasmic Reticulum
FAs	Fatty Acids
FPP	Farnesyl Diphosphate
FT	Fourier Transform
FT-ICR	Fourier Transform- Ion Cyclotron Resonance
FT-IR	Fourier Transform- Infra Red
GC/MS	Gas Chromatography/Mass Spectrometry
GDP	Guanosine Diphosphate
GDR	General Defense Related
GGPP	Geranylgeranyl Diphosphate
GMP	Guanosine Monophosphate
GPP	Geranyl Diphosphate
GRPs	Glycine-Rich Proteins

GTP	Guanosine Triphosphate
H_2O_2	Hydrogen Peroxide
HAI	Hours After Inoculation
$HO^{\cdot}$	Hydroxyl Radical
$HO_2^{\cdot}$	Hydroperoxyl Radical
HPLC	High Performance Liquid Chromatography
HR	Hypersensitive Response
HRGPs	Hydroxyproline-Rich Glycoproteins
ICR	Ion Cyclotron Resonance
IP_3	Inositol1,4,5-Triphosphate
IPP	Isopentenyl Diphosphate
IT	Ion Trap
JA	Jasmonic Acid
KEGG	Kyoto Encyclopedia of Genes and Genomes
LAR	Local Acquired Resistance
LC/NMR/MS	Liquid Chromatography/nuclear magnetic resonance/ mass spectrometry
LG	Linkage Group
LGR	Lesion Growth Rate
MIAMET	Minimum Information About Metabolomics
MS	Mass Spectrometry
MSFACTs	Metabolomics Spectral Formatting and Conversion Tools
MSTFA	N-methyl-N-(trimethylsilyl)trifluoroacetamide
MVOC	Microbial Volatile Organic Compounds

NADPH	Nicotinamide adenine dinucleotide
NC-IUBMB	International Union of Biochemistry and Molecular Biology
NIST	National Institute of Standards and Technology
NMR	Nuclear Magnetic Resonance
$O_2^{\cdot-}$	Superoxide Anion Radical
OAA	Oxaloacetate
OAs	Organic Acids
PAL	Phenylalanine Ammonia-Lyase
PLA	Phospholipase A
PC	Principle Components
PCR	Polymerase Chain Reaction
PI	Phosphatidylinositol
PIP	Phosphatidylinositol Monophosphat
PIP2	Phosphatidylinositol Bisphosphate
PR	Pathogenesis Related
PRD	Pathogenesis Related Decreased in abundance
PRPs	Proline-Rich Proteins
PRI	Pathogenesis Related Increased in abundance
PVA	Potato Virus A
PVM	Potato Virus M
PVS	Potato Virus S
PVY	Potato Virus Y
QTLs	Quantitative Trait Loci

Q-TOF	Quadrupole-Time of Flight
RFLP	Restriction Fragment Length Polymorphism
RGI	Rhamnogalacturonan I
RGII	Rhamnogalacturonan II
RI	Retention Index
mRNA	Messenger Ribonucleic Acid
ROS	Reactive Oxygen Species
RSDR	Resistance Specific Defense Related
RT	Retention Time
SA	Salicylic Acid
SAR	Systemic Acquired Resistance
SNK	Student-Newman-Keuls
SOD	Superoxide Dismutase
SRs	Sugars
SSR	Simple Sequence Repeat
TCA	Tricarboxylic Acid
TL proteins	Thaumatin-like Proteins
TLC	Thin Layer Chromatography
TOF	Time of Flight
UI	Unidentified
Ve	Environmental Variations
Vg	Genetic Variation
vir	Virulent

Vp	Phenotypic Variations

Appendix 3. SAS analysis results of the lesion areas and sporulation amounts

A. Lesion areas (mm^2) measured after 2, 4, 6, and 8 days after the inoculation of the cultivar AC Novachip by *P. infestans.*

AC Novachip	Replicates					
Day No.	**1**	**2**	**3**	**4**	**5**	**Means**
2	70.45	81.01	78.58	68.59	90.17	**77.76** ^{C}
4	126.27	119.22	104.88	90.99	130.29	**114.33** ^{B}
6	110.02	152.92	105.57	113.88	140.06	**124.49** ^{B}
8	180.80	212.43	241.77	222.33	264.06	**224.28** ^{A}

The lesion areas in mm^2 were analyzed using Proc GLM of SAS. ANOVA results showed that the different treatments were significantly different at $P \leq .05$. The means were compared using Duncan's multiple range tests. The means having the same letter are not significantly different at 0.05 levels.

B. The average number of sporangia per a leaflet disc measured 6 days after the inoculation with *P. infestans*. Discs were cut by 18 mm diameter cork borer.

Sporulation disc^{-1}		**Replicates**					**Average**
		1	**2**	**3**	**4**	**5**	
Blocks	**1**	4634	5500	5950	4654	5327	**5213** ^{A}
	2	5625	5850	4428	5488	4623	**5202.8** ^{A}
	3	5410	4730	5324	5388	5856	**5341.6** ^{A}
	4	5563	5650	5769	5902	4834	**5543.6** ^{A}
Gross Average							**5325.3**

Sporangia numbers were subjected to Proc GLM of SAS. ANOVA result showed that the different treatments were not significantly different at $P \leq .05$ and the Pr > F for the replicates and blocks with Pr > 0.7755 and Pr > 0.9712 values, respectively. The gross average number of sporangia was 5.33×10^3 disc $^{-1}$.

Appendix 4. Authorization letters from the co-authors